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Mutations in the p53 tumor suppressor gene occur in roughly 30-40% of all human breast cancers examined to date, indicating that loss of normal function in this gene plays an important role in human breast carcinogenesis. We have chosen to develop and characterize a bitransgenic model, the Wnt-1/p53 mouse, to understand the role of p53 loss in mammary tumor progression. The female Wnt-1/p53 bitransgenic mice are genetically programmed to develop mammary adenocarcinomas by nine months of age. The mice vary only in the status of their inherited p53 genes (two, one, or no intact p53 germline alleles). Thus, these mice represent a powerful model which allows us to study the biological and genetic effects of the presence or absence of p53 on mammary tumor progression in a relatively controlled fashion within a reasonable length of time. Elucidation of the biological processes affected by p53 loss in tumorigenesis may provide important new insights useful in the development of new therapeutic and preventative protocols. Identification of novel genes involved in p53-mediated mammary tumorigenesis may help us to understand the molecular pathways which are important in the loss of mammary epithelial cell growth control.

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Lawrence A. Donehower 10/10/95

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TABLE OF CONTENTS:

Page

1. Front Cover
2. SF 298 Report Documentation
3. Foreword
4. Table of Contents
5. Introduction
6. Body
9. Conclusions
10. References
11. Appendix

Progress Report: The p53-Deficient Mouse as a Breast Cancer Model

Principal Investigator: Lawrence A. Donehower, Ph.D.

INTRODUCTION:

The tumor suppressor gene p53 is the most frequently mutated gene in human cancers. Almost 50% of all human tumors so far analyzed have p53 mutations and 30-40% of mammary adenocarcinomas have associated p53 mutations (1). Even in those mammary tumors with apparent wild type p53, the protein may be abnormally localized or stabilized within the tumor cell (2). In addition to its loss in spontaneously arising breast tumors, inherited p53 gene mutations frequently lead to breast cancers in affected females (3). Thus, loss of normal p53 function is likely to be a critically important event in human breast tumor progression.

While extensive work on the spectrum of p53 mutations in human breast cancers has been performed, our understanding of the role of the biological effects of these mutations in tumor formation and progression is still quite limited. Fortunately, important insights have been derived from molecular studies of wild type p53. Wild type p53 appears to be a checkpoint protein which can arrest cells in late G1 under some circumstances and induce apoptosis under other circumstances (4-6). Agents which cause damage of DNA are potent inducers of p53 and p53 upregulation to cause G1 arrest or apoptosis following DNA damage may be an important aspect of DNA repair and preservation of genomic stability (7).

Aberrant expression of certain oncogenes and tumor suppressor genes also induce high levels of p53 (8,9). When cells are induced to proliferate abnormally by these genes, p53 upregulation may induce apoptosis and thus protect the organism from premature emergence of tumor cell clones. Thus, loss of p53 during progression of a tumor cell clone may result in a failure of that clone to be eliminated by apoptosis and initiate the formation of a viable tumor. Currently, there is significant evidence that attenuated apoptosis due to p53 loss is a rate limiting step in tumor formation (10,11).

While an important effect of p53 loss in tumor progression is abrogated apoptosis, we hypothesize that p53 loss or mutation may have additional important biological effects on mammary tumor formation and progression (12). These are (1) increased rates of cell proliferation independent of apoptotic effects; (2) increased levels of genomic instability in the tumor cells which may lead to further oncogenic lesions; (3) increased rates of angiogenesis in the nascent tumor, allowing a more rapid outgrowth of tumor cells; and (4) increased invasiveness and metastases. All of these biological effects are measurable, and we believe that the p53/Wnt-1 model discussed below is ideal for measuring some of these biological parameters.

Initially, to study the role of p53 in tumorigenesis, we developed a p53-deficient mouse by gene targeting methods in embryonic stem cells (13). These mice contained either one (heterozygotes) or two (nullizygotes) disrupted p53 germ line alleles. In comparison to their normal littermates, the p53-deficient mice showed accelerated tumorigenesis. Half of all p53 heterozygotes developed tumors by 18 months of age,

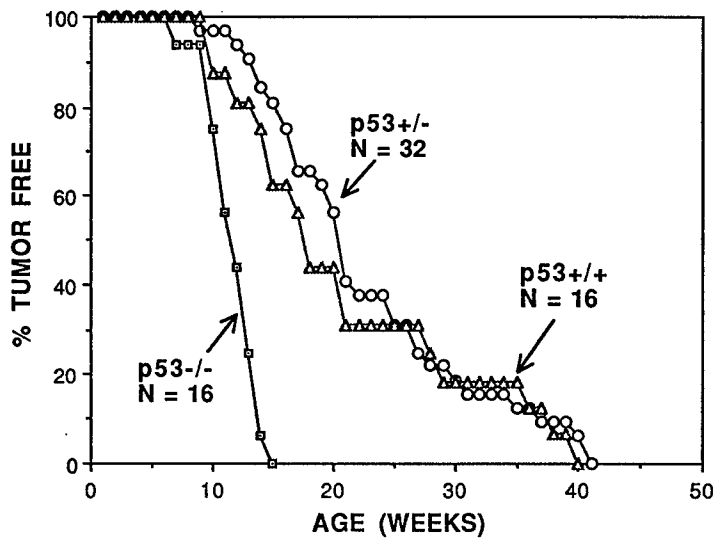
while 100% of nullizygous animals developed tumors by 10 months of age (14). The spectrum of tumors were quite varied in the mice, though lymphomas and sarcomas were most frequently observed (14).

While the p53-deficient mice were useful in exploring the role of p53 loss in tumorigenesis, they possessed certain limitations for mechanistic studies relevant to the Army Breast Cancer Program. First, the p53-deficient mice rarely developed mammary adenocarcinomas (14). Second, the tumor spectrum was quite variable, which complicates mechanistic studies if one assumes that different tumor types have different pathways to tumor formation. And third, the time to tumor development could be quite great (over 12-18 months for many of the p53 heterozygous animals) (14). To eliminate the above problems, we crossed the p53-deficient mice to another mammary tumor-susceptible transgenic line, the Wnt-1 transgenic mouse. Wnt-1 transgenic females, which contain a mouse mammary tumor virus long terminal repeat promoter driving the Wnt-1 oncogene, specifically develop mammary adenocarcinomas before 12 months of age in a stochastic manner (15). Thus, in the Wnt-1/p53 animals, we predicted that virtually all of the females would develop mammary adenocarcinomas either in the presence or absence of p53. Such mice would be ideal for exploring the biological and genetic effects of p53 presence and absence in mammary tumor formation and progression.

BODY:

Following crosses of the p53-deficient mice to the Wnt-1 transgenic mice, we obtained 12 categories of bitransgenic mice which varied according to Wnt-1 transgene status (present or absent), p53 status (p53+/+, p53+/-, or p53-/-), or sex. At least 16 animals of each type were monitored for tumor formation. Only the six categories containing the Wnt-1 transgene developed mammary adenocarcinomas and we will discuss only the females of each p53 genotype which were positive for the transgene. The mammary tumor incidence of the Wnt-1 females is shown below in Fig. 1 for each of the three p53 genotypes:

MAMMARY TUMORIGENESIS IN WNT-1 FEMALES

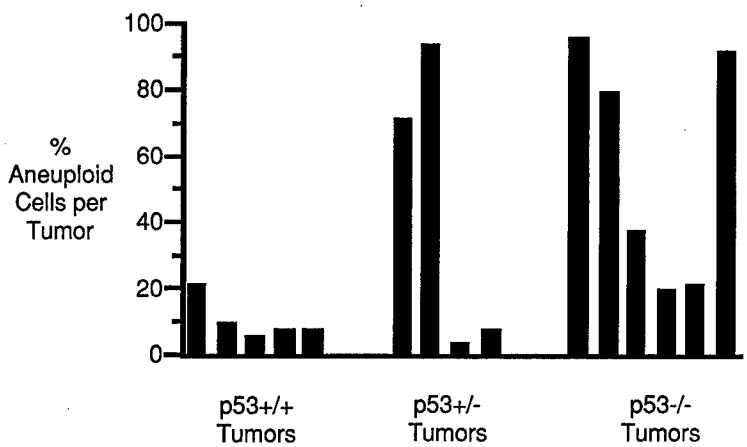


Note that p53+/+ and p53+/- Wnt-1 females develop mammary tumors at approximately same rate, while p53-/- females develop mammary tumors much more rapidly. Interestingly, about half of the p53+/- Wnt-1 transgenic females show loss of the remaining wild type p53 allele, suggesting that there is selection for this event at some time during tumor progression, yet it does not accelerate the average time to tumor appearance.

When we compared the histopathology of the mammary adenocarcinomas, we found that the loss or absence of p53 had profound affects on the cellular morphology within the tumors. p53+/+ and p53+/- tumors which retained their wild type p53 showed more organized cell structures with increased stromal involvement. In contrast, p53-/- tumors and p53+/- tumors which lost their wild type alleles showed a highly anaplastic, disorganized structure with little stromal involvement (data not shown).

Previous in vitro studies had shown that loss of p53 correlated with genomic instability (16,17). In an attempt to study the effects of p53 loss on genomic instability in a mammary tumor model, we examined our tumors for chromosomal complement by two techniques, standard cytogenetics and comparative genomic hybridization. Analyzing 25-50 metaphase spreads for 15 different mammary tumors, we found that p53-containing tumors tended to have diploid chromosome numbers, while mammary tumors with no p53 tended to be quite aneuploid in chromosome numbers. The summary of the cytogenetics data is shown below in Fig. 2:

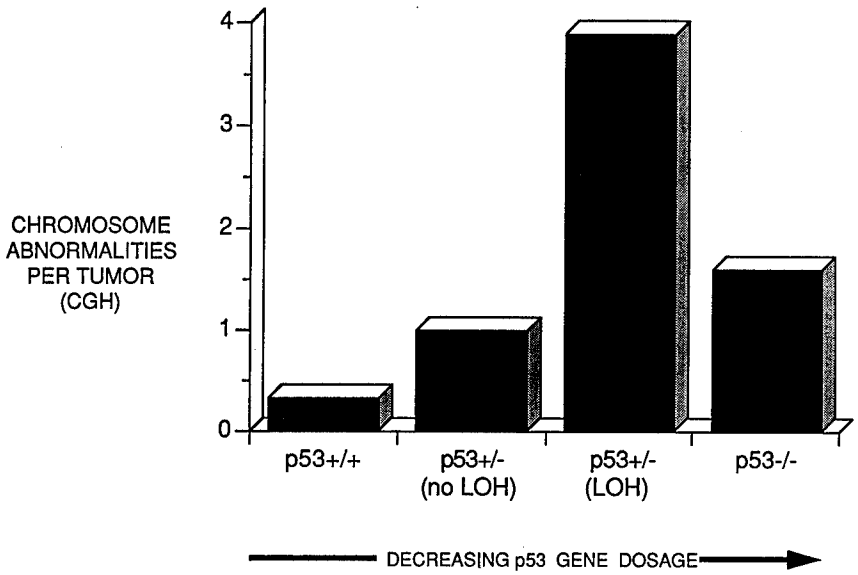
ANEUPLOIDY IN p53/WNT-1 MAMMARY TUMORS



Note that the p53+/- tumors were either highly aneuploid or highly diploid. The aneuploid p53+/- tumors were those that lost their wild type p53 allele, while the diploid p53+/- tumors were those that retained their wild type p53 allele.

The comparative genomic hybridization (CGH) data on the mammary tumors also confirmed the earlier cytogenetic data. This technique uses sophisticated laser monitoring devices to measure the relative copy number within each chromosomal region of a particular tumor DNA (18). This technique also demonstrated that the tumors

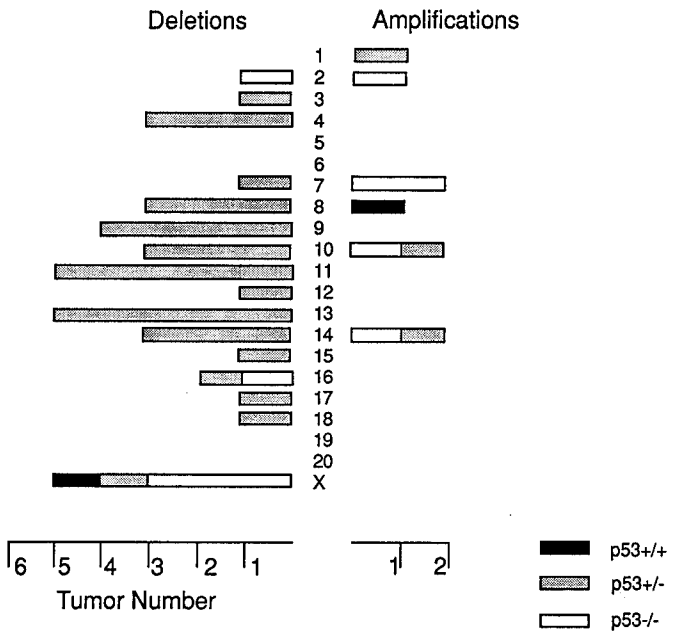
missing p53 showed the most chromosomal abnormalities compared to those mammary tumors retaining wild type p53. The summary of the CGH data for each p53 genotype is shown below in Fig. 3:



Note that the average number of chromosomal abnormalities increases with decreasing p53 gene dosage and these differences are statistically significant by t-test. The exception to this trend is that p53+/- tumors which lose their wild type p53 allele are even more genomically unstable than p53-/- tumors. The reason for this remains unclear.

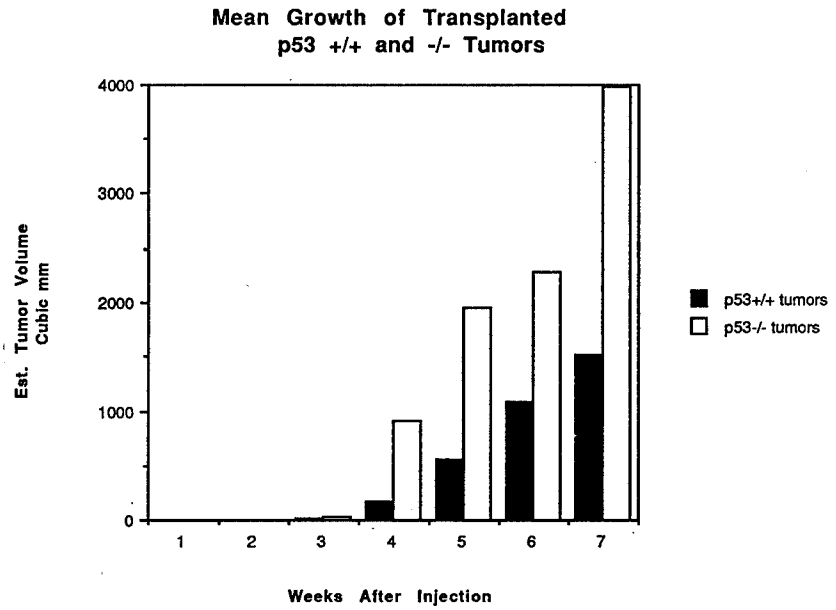
The compilation of the CGH abnormalities showed that the genomic instability in the p53-deficient tumors was non-random in nature. There were certain "hot-spot" mouse chromosomes which were preferentially affected during mammary tumorigenesis and the regions of chromosomal change are shown in graphic form below in Fig. 4:

Wnt-1/p53 Tumors - CGH Chromosomal Abnormalities



The non-random nature of chromosomal changes argues that some of these are selected for during tumor progression and that the observed genomic instability may have important effects on the rate of tumor progression.

The tumor incidence curves shown in Fig. 1 for our bitransgenic model measure the time to first appearance of a visible tumor. Such curves probably reflect primarily early events in tumor formation. However, we wanted to more effectively measure the rates of tumor growth once the tumor is first observed. To do this, we measured the rate of tumor volume growth over time after initial observation for each genotype. We found that while the rate of tumor volume growth for each p53 genotype was quite variable, the average growth of tumors missing p53 was faster than those containing p53. However, to reduce the number of variables inherent in this kind of gross measurement, we harvested 10^5 tumor cells from each tumor and injected them into the same inguinal mammary fat pad of genetically identical recipient SCID mice. The transplanted tumor cells were then monitored weekly for tumor outgrowth. The graphs shown below in Fig. 5 illustrate that p53-deficient tumors grow significantly faster than p53-containing tumors.



Our future goals are to determine the biological mechanisms for this increased rate of tumor growth in tumors lacking intact p53.

CONCLUSIONS:

The data presented in the body of this report argue persuasively that the loss of p53 before or during mammary tumor progression has important effects on the rate of formation and progression of the mammary tumor. Tumor cell histopathology, time to tumor appearance, rate of tumor growth, and genomic instability at the chromosomal and subchromosomal level are all affected by p53 loss. We feel that these results in the first year of the grant period validate the bitransgenic p53/Wnt-1 mouse model as a very

powerful tool for analyzing the role of p53 loss in the mammary tumorigenesis process. The primary goals of the remaining years will be to extend these studies by looking at other potential genetic and biological mechanisms by which p53 loss accelerates tumor progression. Particular attention will be paid to (1) cell cycle regulation and cell proliferation; (2) angiogenesis; and (3) invasiveness and metastases. The particular genes which regulate these biological processes will also be of interest to us and their expression in the presence and absence of p53 during tumor development will be examined closely.

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APPENDIX:

Two reprints from scientific journals which describe some of the work outlined above are appended here. These are:

(1) Donehower, L.A., Godley, L.A., Aldaz, C.M., Pyle, R., Shi, Y.-P., Pinkel, D., Gray, J., Bradley, A., Medina, D., and Varmus, H.E. (1995). Deficiency of p53 accelerates mammary tumorigenesis in Wnt-1 transgenic mice and promotes chromosomal instability. Genes & Development 9:882-895.

(2) Varmus, H.E., Godley, L.A., Roy, S., Taylor, I.C.A., Yuschenkoff, L., Shi, Y.-P., Pinkel, D., Gray, J., Pyle, R., Aldaz, C.M., Bradley, A., Medina, D., and Donehower, L.A. (1995). Defining the steps in a multistep mouse model for mammary carcinogenesis. Cold Spring Harbor Symposium on Quantitative Biology 59:491-499.

Deficiency of *p53* accelerates mammary tumorigenesis in *Wnt-1* transgenic mice and promotes chromosomal instability

Lawrence A. Donehower,[1] Lucy A. Godley,[2] C. Marcelo Aldaz,[3] Ruth Pyle,[1] Yu-Ping Shi,[4] Dan Pinkel,[4] Joe Gray,[4] Allan Bradley,[5] Daniel Medina,[6] and Harold E. Varmus[2,7]

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Deficiency of *p53* accelerates mammary tumorigenesis in *Wnt-1* transgenic mice and promotes chromosomal instability

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By crossing mice that carry a null allele of *p53* with transgenic mice that develop mammary adenocarcinomas under the influence of a *Wnt-1* transgene, we have studied the consequences of p53 deficiency in mammary gland neoplasia. In *Wnt-1* transgenic mice homozygous for the *p53* null allele, tumors appear at an earlier age than in animals heterozygous or wild-type at the *p53* locus. About half of the tumors arising in *p53* heterozygotes exhibit loss of the normal *p53* allele, implying selection for p53-deficient cells. Mammary tumors lacking p53 display less fibrotic histopathology and increased genomic instability with aneuploidy, amplifications, and deletions, as detected by karyotype analysis and comparative genomic hybridization. In one tumor, the amplified region of chromosome 7 had an ectopically expressed *int-2/FGF3* proto-oncogene, a gene known to cooperate with *Wnt-1* in the production of mammary tumors. These findings favor a model in which p53 deficiency relaxes normal restraints on chromosomal number and organization during tumorigenesis.

[*Key Words*: *p53*; *Wnt-1*; mammary tumors; genomic instability; mouse; tumor model]

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The *p53* tumor suppressor gene is the known gene mutated most frequently in human cancers, with deletions and point mutations observed in almost half of all tumors and in about one-fourth of all sporadic breast cancers (Greenblatt et al. 1994). In addition to their frequent occurrence in spontaneously arising tumors, *p53* mutations have also been identified in the germ line of some individuals with an inherited cancer predisposition called Li–Fraumeni syndrome (Malkin et al. 1990; Srivastava et al. 1990). The presence or absence of *p53* mutations in a human tumor may have important clinical implications. A number of studies have shown that tumors missing wild-type *p53* are likely to have a relatively poor prognosis (Callahan 1992; Thor et al. 1992). Moreover, tumors with mutant *p53* appear to be significantly more resistant to the effects of radiation and other anticancer drugs (Lowe et al. 1993a, 1994).

The importance of *p53* loss in cancer is illustrated further by genetically engineered mice that contain one or two defective germ-line *p53* alleles (Donehower et al. 1992; Jacks et al. 1994; Purdie et al. 1994). Mice with two defective *p53* alleles (*p53* − / −) are developmentally normal, but all succumb to tumors by the age of 10 months (Donehower et al. 1992; Harvey et al. 1993a,b; Jacks et al. 1994; Purdie et al. 1994). Mice with a single defective *p53* allele (*p53* + / −) acquire tumors at a later age, but by 18 months half of these mice have developed cancer (Harvey et al. 1993a). Lymphomas and sarcomas are the most frequently observed tumor types in the p53-deficient animals, whereas carcinomas (including mammary adenocarcinomas) are only seen infrequently.

We have now developed an experimental model to study the role of p53 in mammary tumorigenesis by crossing mice that carry a disrupted *p53* allele with transgenic mice programmed to express a proto-oncogene in the mammary gland. We show that the biological and genetic properties of mammary tumors initiated by ectopic expression of *Wnt-1* are dramatically influenced by the presence or absence of p53. Mammary adenocarcinomas appear earlier in both male and female *p53* − / − animals. About half of the tumors arising in *p53* heterozygotes show loss of heterozygosity, although they

are not detected earlier than tumors that retain wild-type *p53*. p53-deficient tumors differ histologically from p53-containing tumors and exhibit several manifestations of genomic instability: aneuploidy in metaphase spreads and genomic regions of increased and decreased DNA sequence copy number as seen by comparative genomic hybridization. These results with transgene-induced cancers in animals extend previous studies with cultured cell lines (Bischoff et al. 1990; Livingstone et al. 1992; Yin et al. 1992) to support a model for p53 as a cell-cycle checkpoint protein whose loss leads to karyotypic instability and cancer.

Results

Mammary tumors are detected earlier in Wnt-1 *transgenic animals lacking a normal* p53 *gene*

To determine the effect of germ-line loss of *p53* alleles on *Wnt-1*-induced mammary tumorigenesis, we crossed p53-deficient mice with *Wnt-1* transgenic mice (*Wnt-1 TG*). The transgene in the *Wnt-1 TG* mice mimics a mouse mammary tumor virus (MMTV) insertion at the *Wnt-1* locus, with the MMTV long terminal repeat (MMTV LTR) in the opposite transcriptional orientation to the mouse *Wnt-1* gene. Previous studies have verified ectopic expression of the *Wnt-1* transgene in the mammary gland, accompanied by marked mammary gland hyperplasia in both female and male transgenic animals (Tsukamoto et al. 1988; Kwan et al. 1992). Because of the hyperplasia in the *Wnt-1 TG* female mammary glands, the animals are unable to nurse their pups. For this reason, we used only *Wnt-1 TG* males in crosses to the p53-deficient mice. Following the crosses, 12 categories of animals were maintained on the basis of different combinations of genotypes: males and females that carry or lack the *Wnt-1* transgene and exhibit one of the three possible *p53* genotypes (+/+, +/−, or −/−). At least 16 virgin animals in each category were maintained for up to 1 year of age while being monitored weekly for tumors (see Materials and methods).

No mammary adenocarcinomas were observed in any males and females without the *Wnt-1* transgene, regardless of *p53* genotype. As expected, nontransgenic *p53*−/− animals developed nonmammary tumors within the first several months of life and all succumbed to cancer by 9 months of age, consistent with previous experience in other genetic backgrounds (Harvey et al. 1993a,b; Jacks et al. 1994; Purdie et al. 1994). In addition, 4 of 27 *p53*+/− mice developed nonmammary tumors by 1 year of age at a rate similar to that observed previously (Harvey et al. 1993a). No tumors of any kind were observed in the nontransgenic *p53*+/+ animals.

All of the female mice containing the *Wnt-1* transgene developed mammary adenocarcinomas by 41 weeks of age (Fig. 1A). Tumors were detected in *Wnt-1 TG p53*+/+ females at a median age of 22.5 weeks, a rate similar to that observed previously for *Wnt-1 TG* females (Tsukamoto et al. 1988; Kwan et al. 1992; Shackleford et al. 1993). Likewise, tumors appeared in the *Wnt-1 TG*

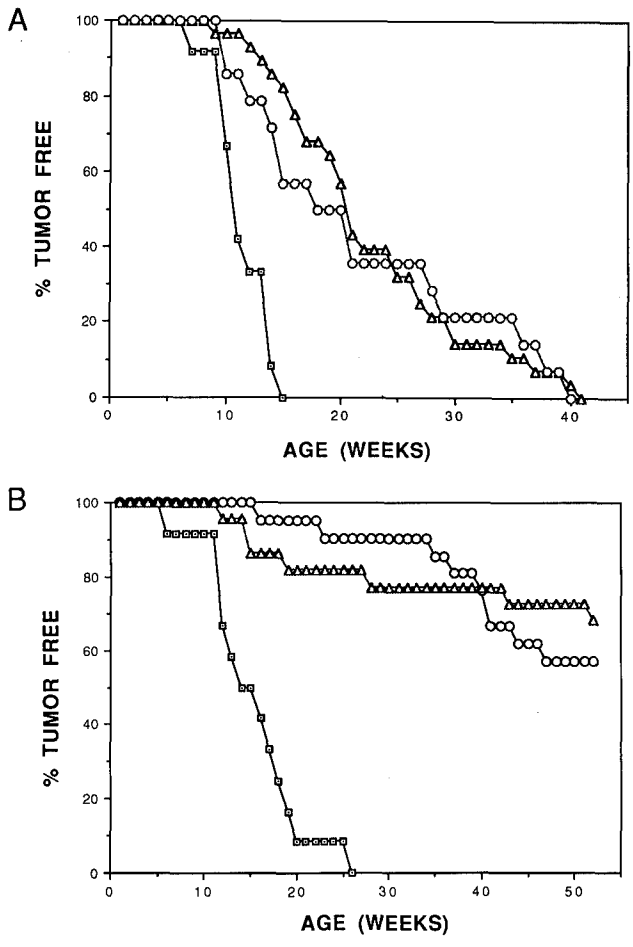

Figure 1. Tumor incidence in *Wnt-1 TG* mice with wild-type and defective germ-line *p53* alleles. The percentage of animals in each group free of palpable tumors (~0.5 cm in diam.) was plotted at weekly intervals. (*A*) *Wnt-1 TG* females. Sixteen *Wnt-1 TG p53*−/− (□), 32 *Wnt-1 TG p53*+/− (△), and 16 *Wnt-1 TG p53*+/+ (○) virgin females were monitored for mammary adenocarcinomas. (*B*) *Wnt-1 TG* males. Twelve *Wnt-1 TG p53*−/− (□), 22 *Wnt-1 TG p53*+/− (△), and 21 *Wnt-1 TG p53*+/+ (○) males were monitored for mammary tumors up to 1 year of age.

p53+/− females with a median time of 23 weeks, suggesting that inheritance of a single normal *p53* allele does not confer any increase in susceptibility to tumor formation. In contrast, tumors were detected significantly earlier in *Wnt-1 TG* females with two defective germ-line *p53* alleles (Fig. 1A). All of the females with the *Wnt-1 TG p53*−/− genotype developed at least one mammary tumor between the ages of 7 and 15 weeks, with a mean time of 11.5 weeks.

Nine of 21 (43%) *Wnt-1 TG p53*+/+ males and 7 of 22 (32%) *Wnt-1 TG p53*+/− males developed mammary tumors by the age of 1 year (Fig. 1B), proportions slightly greater than observed for *Wnt-1 TG* males in previous studies (Tsukamoto et al. 1988; Kwan et al. 1992; Shackleford et al. 1993). The Kaplan–Meier plots are not superimposable for these two cohorts, but the rates of ap-

pearance of tumors were judged to be not significantly different by the generalized Wilcoxon test. The male *Wnt-1 TG p53−/−* animals, however, displayed a dramatic increase in the rate of appearance of mammary tumors compared with their *Wnt-1 TG* p53+/− and *Wnt-1 TG* p53+/+ counterparts (Fig. 1B). Of 16 of these animals, 12 developed mammary tumors by 6 months of age, whereas the other 4 succumbed to thymic lymphomas within the first 6 months, precluding adequate evaluation for mammary tumors. (These latter animals are not included in Fig. 1B.) For those *Wnt-1 TG* p53−/− males that developed mammary adenocarcinomas, the median time required to form a detectable tumor was 15.5 weeks, nearly as short as the median time for appearance of mammary tumors in *Wnt-1 TG p53−/−* females.

Analysis of the wild-type p53 *allele(s) in mammary tumors from* Wnt-1 TG p53+/− *and* Wnt-1 TG p53+/+ *mice*

p53 allele loss accompanied by point mutation in the remaining *p53* allele is a frequent occurrence in human tumors, and we and others have observed loss of the normal *p53* allele when mesenchymal tumors and lymphomas arise in *p53+/−* mice (Harvey et al. 1993a; Jacks et al. 1994; Purdie et al. 1994). To detect loss of heterozygosity (LOH) of the wild-type allele during the development or progression of mammary tumors in *Wnt-1 TG p53+/−* mice, we analyzed the structure of the intact *p53* allele in those tumors by Southern blot hybridization. Of 18 tumors from *Wnt-1 TG p53+/−* females, 8 showed virtually complete loss of the remaining wild-type *p53* allele, whereas the other 10 tumors contained the normal allele (Fig. 2). In two of these latter tumors, comparison of the hybridization intensities of the normal and mutant *p53* alleles suggests that the normal *p53* allele is lost from some tumor cells but retained in others. Four tumors from male *Wnt-1 TG p53+/−* animals were also tested: Two lacked the wild-type allele entirely, and it was underrepresented in a third (data not shown). In tumors from *Wnt-1 TG p53+/−* animals that displayed *p53* LOH, comparison of hybridization to the mutant allele and the *p53* pseudogene suggested that the mutant allele was not duplicated in tumors from either females and males (Fig. 2; data not shown).

The appearance of LOH in a high proportion of cells from about half of the tumors in heterozygous animals indicated that cells lacking an intact *p53* gene might have a selective growth advantage. However, when we replotted the data shown in Figure 1A to separate tumors with LOH from those without LOH, p53 deficiency did not correlate with the detection of tumors at an earlier age. On average, tumors with LOH were detected slightly later than those that retained a normal *p53* allele (data not shown).

To determine whether point mutations had occurred in the wild-type *p53* allele of the *Wnt-1 TG p53+/−* tumors that had not undergone loss of heterozygosity, we sequenced nearly the entire coding region of *p53* cDNAs from four of these tumors (see Materials and methods). Because of the PCR primers used, the first 18 bp and the last 21 bp of the *p53*-protein coding sequences could not be determined, but the rest of the sequence was wild-type (data not shown).

Southern blot analyses and previous sequencing of *p53* cDNAs derived from mammary tumor RNAs from *Wnt-1 TG* mice (V. Pecenka and H.E. Varmus, unpubl.) failed to reveal any changes in the *p53* gene when both *p53* alleles are intact in the germ line.

All of the mammary tumors are adenocarcinomas, but they differ in the degree of fibrosis depending on p53 status

We performed standard histopathological examination on most of the mammary tumors in the study. A total of 40 tumors from female mice and 18 tumors from male mice were evaluated for general histoarchitecture and mammary tumor classification, without prior knowledge of *p53* genotype. The majority of the tumors in female mice (29/40) and in male mice (11/18) were type-B mammary adenocarcinomas (Sass and Dunn 1979). This pathological type was the most common regardless of the *p53* genotype or sex of the animal. The rest of the tumors were either papillary cystic adenocarcinomas or type-A adenocarcinomas, two morphological variants that are more differentiated and less aggressive than the type B adenocarcinomas.

Extensive fibrosis was observed in most of the tumors arising in the *Wnt-1 TG p53+/+* female mice (Fig. 3A). In contrast, tumors from *Wnt-1 TG p53−/−* females did

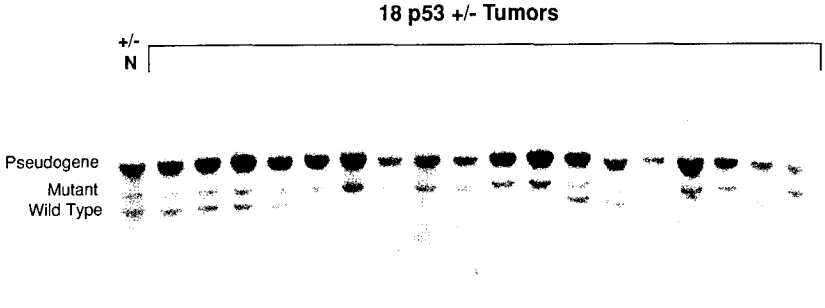

Figure 2. Loss of the wild-type allele in some *Wnt-1 TG p53+/−* tumors. Each lane contains 10 μg of tumor DNA from individual female *Wnt-1 TG p53+/−* tumors cleaved with *Bam*HI. After agarose gel electrophoresis, the DNAs were transferred to nylon and hybridized to the *p53* cDNA exon 2–6 probe. The relevant *Bam*HI fragments are ~5 kb (from the wild-type allele), 6.5 kb (from the null allele), and 10 kb (from the *p53* pseudogene). Lane N contains tail DNA from one of the tumor-bearing animals.

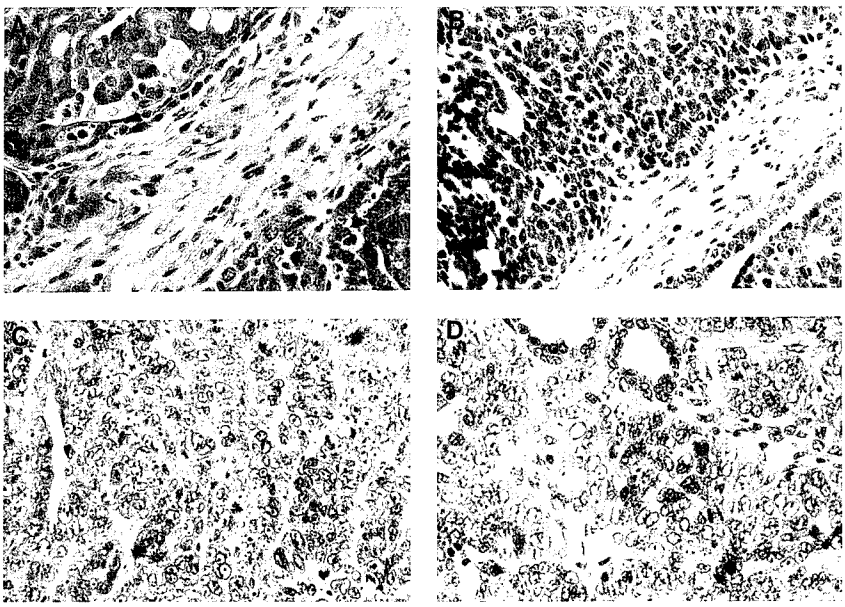

Figure 3. Fibrotic and nonfibrotic histopathology in mammary tumors from mice with different *p53* genotypes. (*A*) Histopathology section of a tumor from a *Wnt-1 TG p53+/+* female. (*B*) Histopathology section of a tumor that retained the wild-type *p53* allele from a *Wnt-1 TG p53+/−* female. (*C*) Histopathology section from a tumor that had lost the wild-type *p53* allele from a *Wnt-1 TG p53+/−* female. (*D*) Histopathology section of a tumor from a *Wnt-1 TG p53−/−* female. All slides are stained with hematoxylin and eosin. Magnification, 94×.

not exhibit significant fibrosis and frequently contained multiple mitotic cells and signs of anaplasia (Fig. 3D). Similar trends were apparent in the smaller collection of tumors from male mice (data not shown). Tumors from *Wnt-1 TG p53+/−* females varied with respect to the degree of fibrosis and number of mitotic cells. When some of these tumors were assessed for loss of the wild-type allele, as illustrated in Figure 2, retention of the wild-type allele correlated quite well with more extensive fibrosis and infrequent mitotic cells (Fig. 3B,C). Of 19 mammary adenocarcinomas with wild-type *p53* that were examined (*p53+/+* and *p53+/−* without LOH), 14 showed extensive fibrosis. In contrast, in tumors without wild-type p53 (*p53−/−* and *p53+/−* with LOH), only 1 of 12 tumors exhibited fibrosis. These findings suggest that significant biological differences may exist between those tumors that produce and those that do not produce p53 protein.

Metaphase spreads reveal a tendency to aneuploidy in p53-deficient mammary tumor cell explants

Earlier studies have revealed that p53-deficient cells in culture exhibit genomic instability as manifested by aneuploidy and susceptibility to gene amplification (Livingstone et al. 1992; Yin et al. 1992). To determine whether partial or complete loss of *p53* predisposes cells to aneuploidy during tumorigenesis in vivo, we examined the karyotypes of cultured cells from five mammary tumors from *Wnt-1 TG p53+/+* females, four mammary tumors from *Wnt-1 TG p53+/−* females, and six mammary tumors from *Wnt-1 TG p53−/−* females. Karyotyping was performed on cells grown in culture for 24–48 hr after dispersion from mammary tumors. Cultures were incubated with Colcemid, fixed, and stained according to standard procedures (Fig. 4I,J), and the chromosomes of 25–50 metaphase spreads were counted for each tumor. Any chromosome count other than 40 (the mouse diploid chromosome number) was considered aneuploid. Chromosome numbers are given in Figure 4 for all cells analyzed from eight representative tumors. Overall, the results demonstrate that the absence of *p53* predisposes tumor cells to aneuploidy.

Cells from *Wnt-1 TG p53+/+* tumors were typically exactly diploid (Fig. 4A,B). In cells from five such tumors, >70% of the metaphase spreads were diploid, and the occasional deviant cells were usually exactly tetraploid or very nearly diploid (Fig. 4A,B; data not shown).

Wnt-1 TG p53−/− tumors fell into two groups on the basis of karyotyping (Fig. 4C,D). Three tumors yielded a very high percentage of cells with grossly abnormal karyotypes, mostly subtetraploid (e.g., Fig. 4C). As is evident from the metaphase spread illustrated in Figure 4J, cells from some of these tumors demonstrated dicentric chromosomes and chromosomes with homogenously staining regions, suggesting gene amplification, in addition to increased chromosome numbers. The other three tumors from *Wnt-1 TG p53 −/−* animals displayed karyotypes similar to those from tumors with a wild-type *p53* genotype (e.g., Fig. 4D). In one case, however, >20% of the cells were tetraploid, whereas in another case nearly 30% of the cells had 39 chromosomes (data not shown). Thus, a deficiency of p53 strongly predisposes cells in mammary tumors to aneuploidy, but is not sufficient to produce it.

We also inspected metaphase spreads from four tumors arising in *Wnt-1 TG p53+/−* animals (Fig. 4E–H). The karyotypes of cells from two of these tumors were not distinguishable from tumors arising in a normal *p53* background. Over 80% of metaphases were diploid, and ~10% were tetraploid with 80 chromosomes (Fig. 4G,H). One of these two tumors retained the normal *p53* allele in most or all cells, but the other had undergone loss of heterozygosity, again demonstrating that the absence of

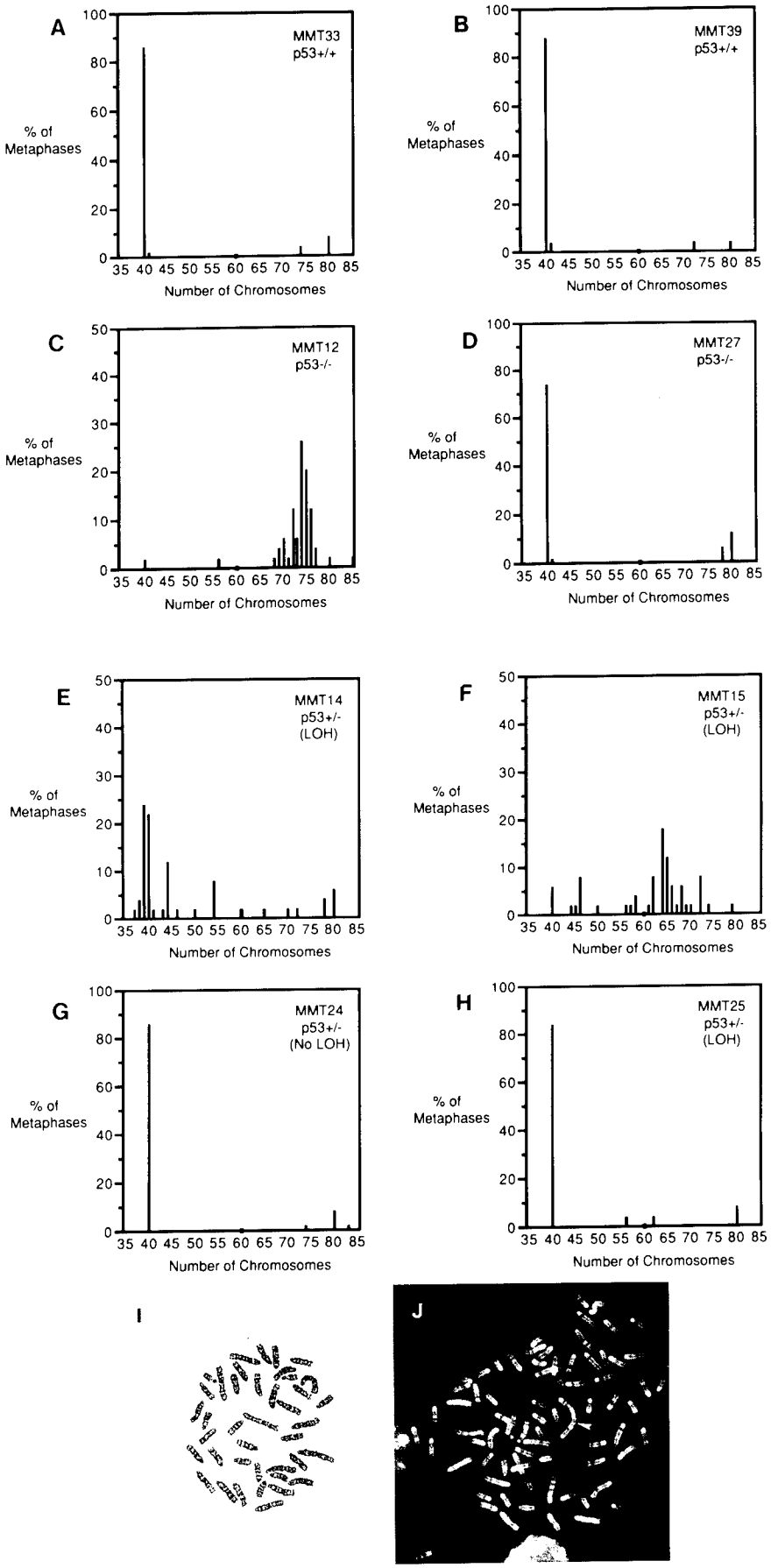

Figure 4. Karyotypes of cultured tumor cells from *Wnt-1 TG* mice with intact or defective *p53* alleles. Metaphase spreads were prepared as described previously (Aldaz et al. 1992). Chromosome numbers were determined in at least 25 different spreads from cultured cells from each tumor. Percentages of tumor cells with given chromosome numbers from representative tumors are shown. (*A,B*) Representative *Wnt-1 TG p53+/+* tumors; (*C,D*) representative *Wnt-1 TG p53−/−* tumors; (*E,F*) tumors from *Wnt-1 TG p53+/−* mice with loss of wild-type *p53* allele; (*G*) tumor from *Wnt-1 TG p53+/−* mouse with retention of the wild-type *p53* allele; (*H*) tumor from *Wnt-1 TG p53+/−* mouse with LOH; (*I*) typical metaphase spread of a tumor cell with a diploid karyotype from a *Wnt-1 TG p53+/+* female photographed after Giemsa trypsin staining; (*J*) typical metaphase spread of tumor cell with a hypotetraploid karyotype from a *Wnt-1 TG p53−/−* tumor photographed after DAPI staining. A dicentric chromosome is indicated by the arrowhead.

p53 from mammary tumor cells does not mandate aneuploidy. The other two *Wnt-1 TG p53+/−* tumors showed extensive aneuploidy, with a wide distribution of chromosome numbers, suggesting that the tumor cell population was heterogeneous and continuing to evolve at the time the animal was sacrificed (Fig. 4E,F). Both of these tumors had lost the wild-type *p53* allele from the majority of cells, as judged by Southern blotting (data not shown), suggesting that the absence of p53 contributes to karyotypic instability.

Comparative genomic hybridization and Southern blot hybridization provide further evidence of genomic instability in p53-deficient tumors

To complement the cytogenetic data and to identify recurring regions of chromosomal change in the *Wnt-1 TG* mammary tumors, we employed the comparative genomic hybridization (CGH) technique (Kallioniemi et al. 1992). This procedure detects regions of increases and decreases in DNA copy number throughout the entire genome of a tumor cell. This is achieved by differential fluorescent labeling of total genomic DNA samples from a tumor and from normal tissue. These two DNAs are hybridized to metaphase spreads of normal mouse chromosomes. Chromosomal regions showing losses and gains in the tumor can be recognized as decreases and increases, respectively, in the intensity of fluorescence from the tumor DNA relative to that from the normal DNA. Two examples of the results of such hybridizations are illustrated in Figure 5. In the first example, the tumor shows many changes by CGH (Fig. 5A,B), and in the other, a single change is observed (Fig. 5C,D). The abnormalities from all of the tumors examined are summarized in Table 1.

Four of the six *Wnt-1 TG p53+/+* mammary tumors examined by CGH had no detectable abnormalities, and the other two had only a single chromosomal alteration. Similarly, two of four tumors from *p53* heterozygous mice that retained the wild-type *p53* allele did not have detectable abnormalities, whereas two others displayed one or three subchromosomal decreases in DNA copy number. These findings are consistent with the low level of aneuploidy observed in such tumors by standard karyotyping.

In contrast, all seven of the *Wnt-1 TG p53−/−* tumors showed at least one chromosomal abnormality, and some exhibited multiple changes. Furthermore, the eight tumors that lost the wild-type *p53* allele while developing in *Wnt-1 TG p53+/−* mice showed the most chromosomal instability. Seven of the tumors displayed at least three regions of DNA gain or loss, and one tumor (W166; Fig. 5A,B) had 10 detectable genetic changes. The *Wnt-1 TG p53+/−* tumors with *p53* LOH averaged 4.2 detectable chromosomal abnormalities per tumor. In contrast, tumors that developed in *Wnt-1 TG p53+/+* mice or in *Wnt-1 TG* 53+/− without LOH averaged only 0.33 and 1.0 abnormalities per tumor. An average of 1.7 chromosomal abnormalities was observed in the tumors from animals with the *Wnt-1 TG p53−/−* genotype. Four of the p53-deficient tumors with CGH abnormalities, two from p53−/− animals and two from p53+/− animals with LOH, were also examined by standard karyotyping. One of each genotype revealed a high level of aneuploidy (see footnotes in Table 1).

Several recurring chromosomal changes were noted by CGH. As expected, many of the *Wnt-1 TG p53+/−* tumors that had lost their wild-type allele showed a loss of DNA from chromosome 11, which contains the mouse *p53* locus (Czosnek et al. 1984; Rotter et al. 1984). Other recurring alterations in the tumors included DNA losses from chromosome 4 (three tumors), chromosome 8 (four tumors), chromosome 9 (four tumors), chromosome 13 (five tumors), and the X chromosome (five tumors). Increases in DNA were also observed for chromosome 7 (two tumors) and chromosome 10 (two tumors).

As an adjunct to CGH, we used genomic Southern blot hybridization with gene-specific probes to ask whether known proto-oncogenes were amplified in *Wnt-1 TG p53*-deficient tumors. Probes for three loci known to be amplified in some human mammary tumors—c-*myc* (Escot et al. 1986), *neu* (Slamon et al. 1987), and *int-2/FGF3* (Lidereau et al. 1988)— were used to test tumor DNAs from 18 *Wnt-1 TG p53−/−* animals and from 6 *Wnt-1 TG p53+/−* animals whose tumors had lost the wild-type *p53* allele (Fig. 6A; data not shown).

In tumor B from animal W177, the *int-2/FGF3* locus was increased to about six to eight copies per cell. This change was consistent with the CGH profile for tumor 177B, which showed only one abnormality: increased representation of DNA from the distal portion of mouse chromosome 7, the chromosomal location of *int-2/FGF3* (Fig. 5C,D). A second tumor that arose independently in this animal (177A) did not show these changes by CGH or Southern blot hybridization (Table 1; Fig. 6A).

In some human breast tumors, the *int-2/FGF3* region is amplified, and the amplified domain includes the linked genes *hst/FGF4* and *PRAD-1/cyclin D1*. Because *PRAD-1/cyclin D1* is the only one of these three loci expressed in such tumors, the *cyclinD1* gene rather than either *FGF* gene is believed to be contributing to carcinogenesis (Lammie et al. 1991). However, several kinds of experiments suggest that ectopic expression of *FGF* genes can synergize with *Wnt-1* to promote mouse mammary tumors (Peters et al. 1986; Kwan et al. 1992; Shackleford et al. 1993). When we measured *int-2/FGF3*, *hst/FGF4*, or *PRAD-1/cyclin D1* RNAs in tumor W177B and in several other tumors, *int-2/FGF-3* RNA was abundant in W177B but undetectable in other tumors (Fig. 6B). Equally low levels of *hst/FGF4* and *PRAD-1/cyclin D1* RNA were observed in all of the samples (data not shown). These findings suggest that activated expression of *int-2/FGF-3* (by an unknown mechanism) accompanied by amplification of the gene (perhaps facilitated by p53 deficiency), participated in the oncogenic process in tumor 177B.

Discussion

Abnormalities of *p53* are common in human breast can-

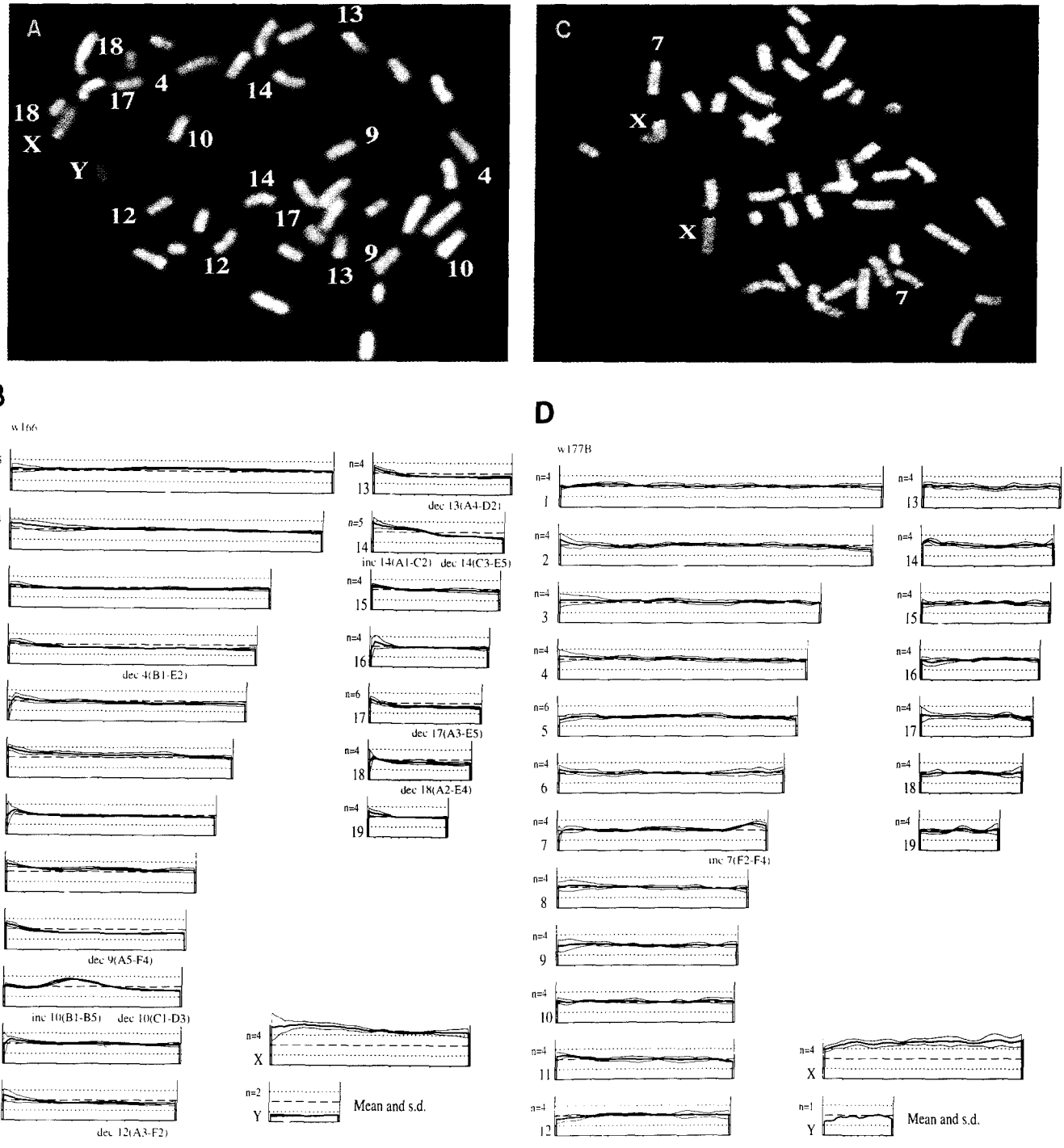

Figure 5. Representative comparative genomic hybridization images and fluorescence ratio profiles. (*A*) Composite digital image from CGH analysis of tumor W166 (*Wnt-1 TG p53+/−* tumor that had lost the wild-type *p53* allele). Tumor genomic DNA hybridization signal is displayed in green, normal genomic DNA in red, and the normal male metaphase target chromosomes in blue. Regions of relatively green fluorescence indicate increased copy number of these sequences in the tumor, and red regions indicate decreases of copy number relative to the average for the tumor. Blue regions of the target chromosomes contain repeated satellite sequences where hybridization is blocked by unlabeled C_0t-1 DNA. Those chromosomes containing regions of variant copy number are labeled in the image. The specimen is from a female mouse, and the normal DNA is male so that the X chromosome appears green and the Y chromosome appears red. (*B*) Profiles of the green to red fluorescence intensity for each of the chromosomes from tumor W166. The heavy lines show the average of profiles from several chromosomes of each type (indicated by the *n* value next to each profile); the thin lines show +/±1 S.D. from the mean. The lengths of the profiles are arbitrarily normalized and are not accurately proportional to chromosome length. The regions of variant copy number are indicated under the individual profiles and correspond to those shown in Table 1. (*C*) Composite digital image from the analysis of a *Wnt-1 TG p53−/−* tumor, W177B. (*D*) Ratio profiles of tumor W177B. The only significant variation is on the distal portion of chromosome 7 corresponding to the green region of the image in *C*.

Table 1. *Comparative genomic hybridization of tumor DNAs*

Wnt-1 tumor genotype	Tumor	Modal karyotype[a]
p53+/+	1269	normal
	1370	normal
	1477	normal
	1498	inc 8(A)
	1562	dec X(D-F)
	MMT19[b]	normal
p53+/−	W15	normal
(no *p53* LOH)	W08A	normal
	W21B	dec 3(E-H); dec 7(C-F); dec 13(A5-D)
	W55	dec 9(B-F)
p53+/−	W32	dec 8(A1-E2); dec 9(E3-F4); dec X(E-F4)
(*p53* LOH)	W108	dec 4(B-E); dec 8(B-E); dec 11
	W139	inc 1(B-H); dec 11; dec 13(A5-D); dec 14(C3-E)
	W165	dec 11(A5-E)
	W166	dec 4(B1-E2); dec 9(A5-F4); inc 10(B1-B5); dec 10(C1-D3); dec 12(A3-F2); dec 13(A4-D); dec 14(C3-E5); inc 14(A1-C2); dec 17(A3-E5); dec 18(A2-E4)
	W126	dec 4(D-E); dec 9; dec 10; dec 11; dec 13; dec 16
	MMT14[c]	dec 8; inc 10; dec 14(C-E); dec 15(D-E)
	MMT25[b]	dec 10(B-D); dec 11(C-E); dec 13(B-D)
p53−/−	W121	dec X
	W180	dec 2(G-H)
	W177A	dec X(D-F)
	W177B	inc 7F
	W154	inc 2H; inc 14B; dec X
	MMT12[c]	inc 10(A-B)
	MMT27[b]	inc 7(A-F); dec 16(C-G)

[a]Tumor DNAs were analyzed by comparative genomic hybridization (Kallioniemi et al. 1992). Chromosomal abnormalities are indicated dec for DNA copy number decreases and inc for DNA copy number increases, followed by the mouse chromosome number. Subchromosomal regions affected by these changes are indicated in parentheses by uppercase letters. Each letter represents a band visible by DAPI staining; A is the band closest to the centromere.
[b]Tumor diploid by karyotyping.
[c]Tumor aneuploid by karyotyping.

cer (Greenblatt et al. 1994), yet mammary tumors have rarely been encountered in mice with null alleles at the *p53* locus (Donehower et al. 1992; Jacks et al. 1994; Purdie et al. 1994) or with mutant *p53* transgenes (Lavigueur et al. 1989). To examine the role of *p53* in mammary tumorigenesis in an animal model, we have introduced a *Wnt-1* transgene, known to induce mammary hyperplasia and a strong predisposition to mammary cancer, into mice bearing one or two copies of a targeted null mutation of *p53*. Two major findings with the progeny of this cross strongly support our conclusion that a deficiency of *p53* influences the formation of mammary tumors in the presence of the *Wnt-1* transgene: (1) Tumors arise much earlier in animals that do not inherit an intact *p53* gene (Fig. 1), and (2) tumors that develop in animals constitutively heterozygous at the *p53* locus often show loss of heterozygosity (Fig. 2), implying a selective growth advantage for mammary tumor cells in which no functional *p53* gene remains.

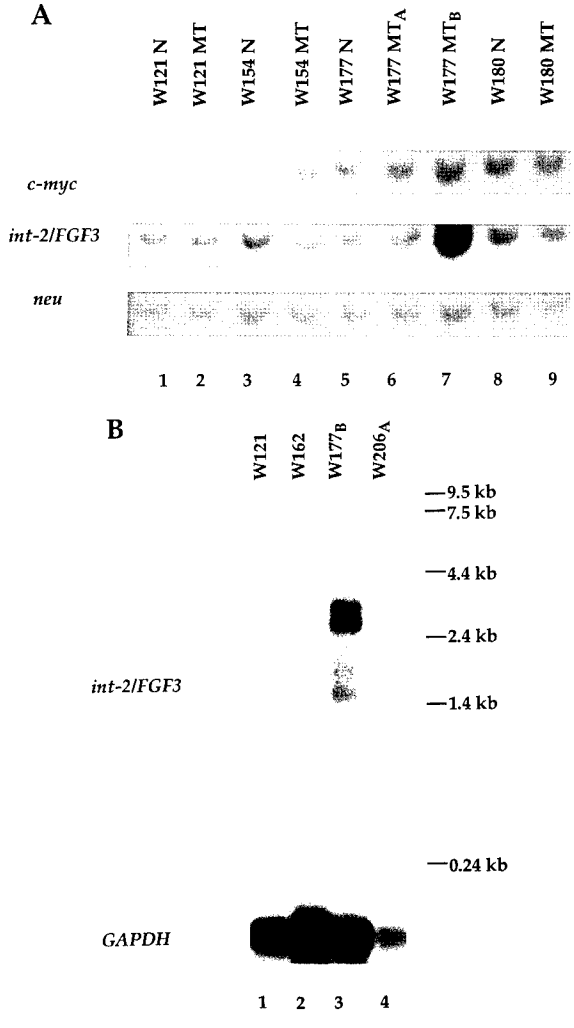

Figure 6. Southern and Northern blotting analyses of *Wnt-1 TG p53*−/− mammary tumors. (*A*) Gene amplification of the *int-2/FGF3* locus in *Wnt-1 TG p53*−/− tumor W177B. Southern blotting was performed with multiple probes on paired samples from *Wnt-1 TG p53*−/− tumors. At the *top* of each lane, the mouse identification number is given: (N) normal tail DNA; (MT), mammary tumor DNA. Subscript letters distinguish independently arising tumors in a single animal. The gene from which each probe is derived is given at *left*. (*B*) Overexpression of *int-2/FGF3* in *Wnt-1 TG p53*−/− mammary tumor W177B. Northern blotting of RNA from *Wnt-1 TG p53*−/− tumors was performed with multiple probes. The gene from which each probe is derived is given at *left*, and RNA sizing is at *right*.

Three striking correlations have emerged from our efforts to understand the contribution that a deficiency of *p53* might make to the oncogenic process in these animals: (1) We noted histopathological differences between tumors that retained and those that lacked a *p53* gene. p53-Deficient tumors were, in general, less fibrotic, and contained more mitotic and anaplastic cells; (2) aneuploidy was frequent in cells derived from tumors lacking *p53* and much less common in tumor cells that retained *p53*; and (3) relative gains and losses of chromosomal DNA, as measured by CGH, were more common in tumors that lacked p53, especially in those p53-deficient tumors resulting from loss of a wild-type *p53* gene in a heterozygous background. These findings imply that there are at least two pathogenic routes by which mammary tumors can arise in *Wnt-1* transgenic mice. In one, p53 continues to function, tumor cells show little aneuploidy or chromosomal rearrangement, and the tumors contain extensive fibrosis. In the other, p53 function is lost, the tumor cell genome is destabilized, and the tumors are minimally fibrotic.

p53 deficiency and the kinetics of tumorigenesis

We initiated the experiments described here in hopes of understanding the multistep process of carcinogenesis in greater detail. Our results have provoked at least two perplexing questions about the number, timing, and functional consequence of the events in mammary tumorigenesis: (1) Why do tumors appear relatively early in p53-deficient mice? and (2) why do tumors fail to appear early in heterozygous mice when the normal *p53* gene has been eliminated? We have considered possible answers to these questions in the context of two models that posit cell-autonomous and non-cell-autonomous functions for p53 protein (Fig. 7A,B).

Loss of one or more of the cell-autonomous functions previously proposed for the *p53* gene product might foster the development of a tumor and thereby help to explain the early onset of tumors in *Wnt-1 TG p53−/−* animals (Fig. 7A): (1) Loss of cell-cycle control and accompanying genomic instability (Livingstone et al. 1992; Yin et al. 1992; Harvey et al. 1993c) might favor the more rapid accumulation of additional mutations necessary for tumor development; (2) changes in control of p53-dependent transcription (Farmer et al. 1992; Kern et al 1992) might affect cell growth rates or other cell processes; (3) resistance to apoptosis (Yonish-Rouach et al. 1991; Clarke et al. 1993; Lowe et al. 1993a,b) might increase cell numbers during tumor growth, because p53-

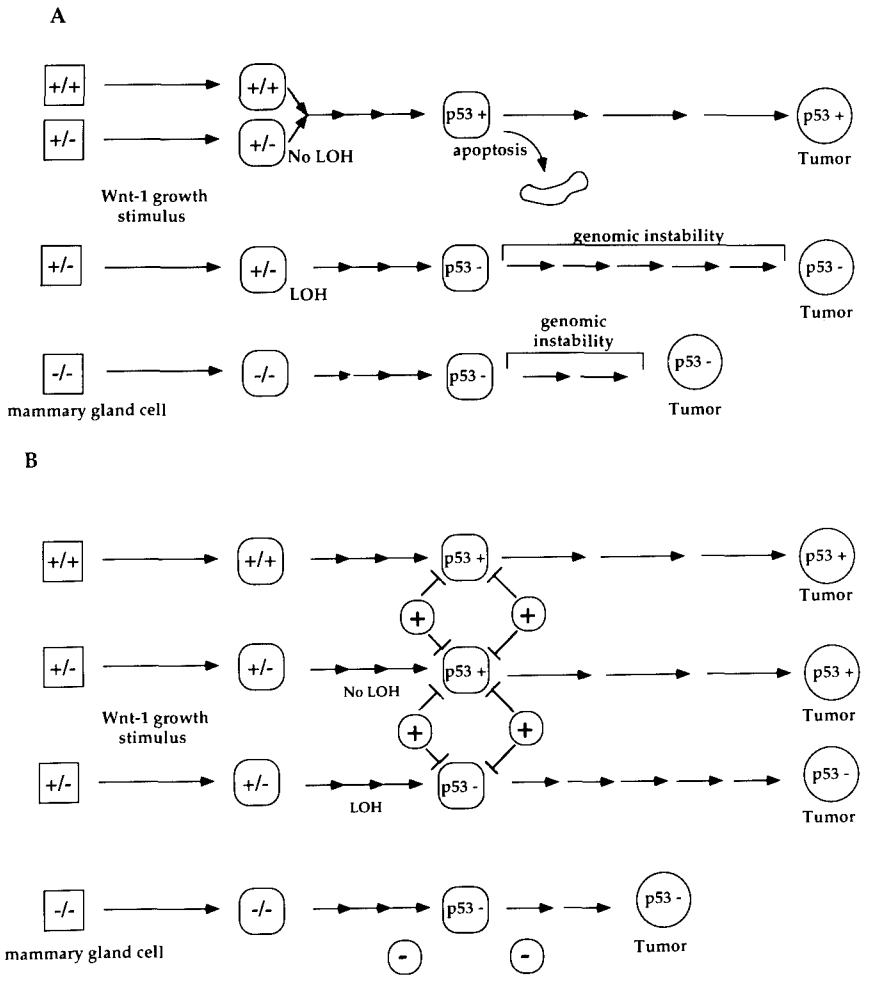

Figure 7. Multistep models of mammary tumorigenesis in *Wnt-1 TG p53* mice. (Squares) Normal mammary gland epithelial cells; (squares with rounded edges) preneoplastic cells; and (circles) the dominant cell population in a tumor. Arrows between cells indicate genetic or epigenetic changes that occur during tumor cell initiation and progression. (*A*) The cell-autonomous model. Initially in all *Wnt-1 TG* mice, ectopic expression of Wnt-1 protein in normal mammary gland cells induces polyclonal hyperplastic growth. Further growth stimulation by Wnt-1 accompanies additional genetic or epigenetic changes that produce clonal tumor growth. In this model the rate of progression depends on the *p53* status of the initiated cell. Cells that retain wild-type *p53* produce tumors at a delayed rate, possibly because of a lower frequency of genetic change and *p53*-mediated apoptosis. (*B*) The non-cell-autonomous model. In this model tumor cell progression is regulated in part by events extrinsic to that cell that will give rise ultimately to a tumor. The *p53* genotype of the host animal, not the genotype of the nascent tumor cell, determines the speed of tumor formation. It is hypothesized that surrounding cells that have wild-type *p53* (*p53+/+* and *p53+/−* animals) inhibit expansion of the tumor cell clone, whereas in *p53−/−* animals this inhibitory effect does not occur. Fewer genetic changes may be necessary for the nascent tumor cell in *p53−/−* animals to form a tumor because of the reduction of inhibition by surrounding cells.

mediated programmed cell death no longer counterbalances Wnt-1-stimulated proliferation. Although we have provided considerable support for the first of these three explanations, we have not yet tested directly for contributions by the latter two. Moreover, we have identified only a single specific mutation, amplification of *int-2/FGF-3* in tumor 177B, that is likely to participate in carcinogenesis, despite abundance evidence for genomic instability.

These proposed effects of p53 on cell growth are not, however, sufficient to account for the observation that *p53* LOH failed to accelerate the development of tumors in *Wnt-1 TG p53+/−* animals. Perhaps the simplest explanation for this observation is that *p53* LOH is a late event in this tumorigenesis model. This hypothesis can be tested further by comparison of the frequency of *p53* LOH as a function of tumor size. A non-cell-autonomous model is presented in Figure 7B to account for the paradoxical finding that *Wnt-1 TG p53+/−* tumors with LOH display more evidence of genomic instability than *Wnt-1 TG p53−/−* tumors. *Wnt-1 TG p53−/−* mice might develop mammary tumors earlier than their *Wnt-1 TG p53+/−* and *Wnt-1 TG p53+/+* counterparts because of a permissive environment afforded by *p53−/−* cells surrounding nascent tumor cells. Conversely, p53-containing cells that surround neoplastic cells, including p53-deficient tumor cells, might provide an inhibitory influence and retard growth of the tumor clone, demanding that the clone undergo further mutations to overcome the inhibition.

The idea that normal cells surrounding a transformed cell can influence its growth potential has been demonstrated previously both in vitro and in vivo, as assayed by focus formation in primary rodent fibroblasts (Land et al. 1983) and by tumorigenic potential of mixtures of normal and preneoplastic mammary cells (Medina et al. 1978). Such a model implies that wild-type p53 may have an extracellular inhibitory role in growth control, in addition to its well-characterized intracellular effects. For example, wild-type p53 was shown to stimulate expression of thrombospondin-1, a potent inhibitor of angiogenesis (Dameron et al. 1994).

p53 and genomic stability

Our findings of aneuploidy and CGH abnormalities in mammary tumors in conjunction with p53 deficiency are consistent with previous reports that the lack of p53 predisposes cultured cells to aneuploidy and amplification of selectable genes (Livingstone et al. 1992; Yin et al. 1992). By extending these observations to a tumor model in an intact animal, we provide support for the hypothesis that p53 deficiency contributes to carcinogenesis by promoting chromosomal rearrangements that favor tumor cell growth. But the findings also emphasize the importance of two largely unresolved questions.

1. What is the mechanism by which p53 protects a cell from genomic instability? p53 is believed to defend cells from premature entry into the S phase of the cell cycle, an idea that has been especially well-documented after exposure of cells to genotoxic agents (Kastan et al. 1991, 1992; Lu and Lane 1993). Failure to block passage through the cell cycle could result in permanent genetic change if the genome has been assaulted by radiation or chemical mutagens, without sufficient opportunity for repair. Deficiency of p53 per se does not produce chromosomal abnormalities because mice carrying targeted germ-line mutations of *p53* can develop normally despite a total absence of p53 protein (Donehower et al. 1992; Jacks et al. 1994; Purdie et al. 1994). Also, some mammary tumors from p53-deficient animals are composed largely of diploid cells with relatively few abnormalities seen by CGH (Fig. 5). However, the absence or loss of p53 causes a predisposition to genomic instability in established cell lines and in cultured fibroblasts from p53-deficient animals (Bischoff et al. 1990; Livingstone et al. 1992; Yin et al. 1992; Harvey et al. 1993c), and we have observed a higher frequency of aneuploidy and abnormalities detectable with CGH in p53-deficient tumors (Figs. 4 and 5; Table 1). The factors that provoke genomic instability in p53-deficient cells remain unknown. Their identity might be useful in considering both the origins and treatment of cancers.

2. What are the specific genetic consequences of p53 deficiency that promote the growth of tumor cells? In at least one instance, tumor 177B, we were able to provide some answer to this question, because the amplified DNA included a gene, *int-2/FGF-3*, that is known from several other kinds of experiments, to collaborate with *Wnt-1* when expressed ectopically in mammary tissue (Peters et al. 1986; Kwan et al. 1992; Shackleford et al. 1993). The increases and decreases of several other chromosomal domains may also be functionally important, because the changes were encountered in multiple tumors or because the domains are syntenic with those implicated in human or experimental tumors. Recurring decreases in DNA occurred on chromosomes 4, 8, 9, 11, 13, and X, arguing that the genomic instability in this model is at least partially nonrandom and may represent genetic events that provide a selective growth advantage during tumor progression. The role such genomic losses play in the progression of mammary cancer remains to be elucidated. However, the largely normal karyological and CGH patterns observed in tumors that retain p53 show that the abnormalities detectable with these methods are not absolutely required for Wnt-1-promoted tumorigenesis. In addition, it should be noted that increased genomic instability did not necessarily correlate with accelerated tumorigenesis, as the *Wnt-1 TG p53+/−* tumors with LOH displayed the greatest numbers of karyotypic abnormalities but did not show an increase in rate of formation in comparison to *Wnt-1 TG p53+/+* tumors and *Wnt-1 TG p53+/−* tumors without LOH. This lack of correlation suggests that genomic instability may provide a selective advantage during tumor progression, but does not necessarily affect the rate at which the tumors appear.

Further definition of multistep mammary tumorigenesis

Our work demonstrates that the proto-oncogene *Wnt-1* and the tumor suppressor gene *p53* can collaborate to produce mammary cancers in mice, but it is evident from the kinetics of appearance of new tumors that other events are required. We presume these to be mutations, at least some of which are represented by the genomic abnormalities observed by CGH. Moreover, we do not know how many mutations are required, how many oncogenic combinations of mutations are possible (even in the limited context of a Wnt-1-stimulated, hyperplastic mammary gland), or what constraints are placed on the order in which the mutations occur.

Multistep carcinogenesis has been documented in some human cancers (Fearon and Vogelstein 1990) and often involves the *p53* gene (Greenblatt et al. 1994). p53 mutations have been associated with genomic instability during tumor development in at least one clinical setting, esophageal cancer (Neshat et al. 1994), although in general human tissues are difficult to obtain at different stages of tumorigenesis in a single patient or in a genetically homogeneous population. For this reason, many laboratories are using mouse models to explore multistep models for carcinogenesis (Cardiff and Muller 1993; Kemp et al. 1993; Christofori and Hanahan 1994). Some of these models incorporate, as ours has done, a gene that is frequently mutated in the same type of cancer in human patients (Dietrich et al. 1993; Oltvai and Korsmeyer 1994). It is evident from recent work with such models that important physiological events—such as augmented angiogenic activity (Kandel et al. 1991; Shing et al. 1993; Fidler and Ellis 1994) or a diminished rate of apoptosis (Symonds et al. 1994)—can have major consequences during tumor progression. The *Wnt-1* transgenic model has special advantages in this regard, because it is possible to examine mammary tissue at several stages of tumor development, ranging from early hyperplasia to metastatic growth (Tsukamoto et al. 1988). It will now be important to ask whether changes in angiogenic and apoptotic activity accompany the changes we have described here in histology, genomic composition, and integrity of specific genes.

Materials and methods

Mice

The *Wnt-1 TG* mice used in the crosses described here were the offspring of two *Wnt-1 TG* males from line 303 described previously (Tsukamoto et al. 1988). These mice were of mixed SJL×C57/BL/6 genetic background. The p53-deficient mice were from a pure 129/Sv line of mice containing one or two germ-line *p53* null alleles (Harvey et al. 1993b). The two *Wnt-1* males were crossed to heterozygous (*p53*+/−) 129/Sv females to derive F_1 mice of four possible genotypes (*p53*+/+; *Wnt-1 TG p53*+/+; *p53*+/−; *Wnt-1 TG p53*+/−). F_1 *p53*+/− females were crossed to F_1 *Wnt-1 TG p53*+/− males to obtain F_2 mice that carried any of the *Wnt-1 TG p53* genotypes found in the F_1 population as well as *Wnt-1 TG p53*−/− or *p53*−/−. To obtain larger numbers of mice with *p53*−/− genotypes with or without the *Wnt-1* transgene, F_2 *p53*−/− females were mated to *Wnt-1 TG p53* −/− males. All of the mice were monitored visually twice weekly for the appearance of tumors for up to 1 year. When a tumor of ~0.5 cm in diameter was detected, the age of the mouse was recorded and used to generate the Kaplan–Meier plots in Figure 1. Once a tumor reached 1.5–2 cm in diameter, the tumor-bearing mouse was sacrificed, and tissue sections removed for histopathology. The remainder of the tumor was frozen at −70°C for nucleic acid analyses.

Nucleic acid isolation and analysis

We determined the *p53* and *Wnt-1* genotypes of the offspring from the crosses by use of methods described previously (Tsukamoto et al. 1988; Donehower et al. 1992; Harvey et al. 1993a). To determine the coding sequence of the remaining *p53* allele in *Wnt-1 TG p53*+/− mammary tumors that did not show LOH, total cellular RNA from a small tissue segment from each of four tumors was prepared using the RNAzol B kit (Tel-Test, Inc.) according to the manufacturer's instructions. We then used reverse transcriptase–PCR (with the RT–PCR kit from Perkin-Elmer Cetus) to amplify the *p53* cDNA from the four tumor RNAs.

Initially, the amplification primers used were derived from exons 4 and 10 of the mouse cDNA, and these amplified exons 5–9 of the murine *p53* gene. The nucleotide sequences of these primers were 5′-CAGTCTGGGACAGCCAAGTC-3′ (exon 4) and 5′-CTCCCGGAACATCTGGAAGC-3′ (exon 10). The amplified exon 4–10 fragments were purified from a low-temperature agarose gel following electrophoresis, treated with Klenow polymerase and T4 polynucleotide kinase, and then ligated into the plasmid cloning vector Bluescript II (Stratagene) at the *Sma*I site. Sequencing was performed on the Applied Biosystems automated sequencer with the SP6 and T7 universal sequencing primers adjacent to the insertion site. Standard dideoxy sequencing with the Sequenase sequencing kit (U.S. Biochemical) was also performed on parts of the cloned cDNA with two internal primers from exons 5 and 9: 5′-CGTGAGACGCTGCCCCCACCATG-3′ (exon 5) and 5′-TTGCGGGGGAGAGGCGCTTGTGC-3′ (exon 9).

Subsequently, fragments containing the remainder of the *p53* coding exons were also obtained by amplifying exons 2–4 and exons 10–11. The sequences of these primers used were 5′-GGAATTCGCCATGGAGGAGTCACAGTCG-3′ (exon 2); 5′-GCAGAATAGCTTATTGAGGGGAGG-3′ (exon 5); 5′-GCGCAAAGAGAGCGCTGCCC-3′ (exon 8–9); 5′-CCCAAGCTTCAGTCTGAGTCAGGCCCCAC-3′ (exon 11).

Karyotype analysis

Cytogenetic preparations were obtained essentially as described in Aldaz et al. (1992).

Comparative genomic hybridization

Comparative genomic hybridization was performed essentially as described in Kallioniemi et al. (1992). Briefly, tumor genomic DNA and normal genomic DNA were labeled with biotin 14 dATP (GIBCO-BRL) and digoxigenin-11-dUTP (Boehringer Mannheim) by nick translation or random priming. Double stranded labeled DNA with a fragment size distribution of 600–1000 bp gave optimal signals if labeled by nick translation. The size distribution was between 150 and 500 bp when random primer labeling was used. Random priming was used in most of this work because it required less input DNA and more reliably produced good hybridization signals. Labeled tumor and normal

genomic DNAs (60–100ng) and unlabeled mouse Cot-1 DNA (GIBCO-BRL) (10–20 μg) were coprecipitated and dissolved in 10 μl of hybridization solution to obtain a final composition of 50% formamide, 10% dextran sulfate, and 2× SSC (pH 7). This mixture was heated to 70°C for 5 min to denature the DNAs and incubated at 37°C for 5–30 min. Normal mouse metaphase chromosomes prepared from fibroblast cultures were denatured at 80–85°C in 70% formamide, 2× SSC, for 5 min and dehydrated through an ethanol series. The hybridization mixture was applied to the slides, the coverslip sealed with rubber cement, and the slide incubated at 37°C for 4–5 days. After hybridization, the slides were washed and stained with a single layer of avidin–FITC (Vector Laboratories) and anti-digoxigenin–rhodamine (Boehringer Mannheim). Slides were counterstained with 0.1–0.2 μM DAPI in an antifade solution. The low DAPI concentration produced sufficient banding to permit identification of the mouse chromosomes.

Digital images of each of the fluorochromes in the specimens were obtained under computer control with a fluorescence microscope equipped with a CCD camera. Profiles of the fluorescence intensities of the normal and tumor DNA hybridization signals, and the intensity ratio profiles were calculated as described in Piper et al. (1995). The profiles were normalized so that the average value was 1.0 for the entire genome. Chromosomal regions where the ratio profile deviated significantly from the average were classified as either increases or decreases in DNA copy number.

Histopathology of tumor tissue

Histopathology of tumor tissues was as described previously (Medina 1973).

Southern blotting analysis of mammary tumors for gene amplification

DNA was isolated from tail and tumor tissue and 5 μg was digested with *Bam*HI as described previously (Donehower et al. 1992; Harvey et al. 1993a). The Southern blot was hybridized sequentially with several probes at the indicated temperatures: the 420-bp *Bam*HI fragment of rat *neu* (Bargmann et al. 1986) at 55°C, the 2.15-kb *Hin*dIII fragment of mouse *int-2/FGF3* (Mansour and Martin 1988) at 65°C, and the 600-bp *Cla*I–*Hin*dIII fragment of human c-*myc* (Stone et al. 1987) at 55°C. Following each hybridization, the blot was stripped of probe as recommended by the manufacturer. Quantitation was performed with a PhosphorImager (Molecular Dynamics).

Northern blotting analysis of mammary tumors

Portions of each tumor were used for RNA isolation by RNazol B reagent (Tel-Test, Inc.) according to manufacturer's protocol. One microgram of poly(-A)$^+$ RNA was electrophoresed through a 1% agarose formaldehyde gel and transfered to Hybond N (Amersham) in 20× SSC. Hybridizations were performed as described above. Additional probes and the corresponding hybridization temperatures used were the 1.7-kb *Bam*HI–*Eco*RI fragment of human *PRAD-1/cyclin D1* (Arnold et al. 1989) at 55°C and the 1.6-kb *Hin*dIII fragment of mouse *hst /FGF4* (Peters et al. 1989) at 65°C. The blot was stripped following each hybridization, as recommended by the manufacturer and as confirmed by autoradiography.

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Defining the Steps in a Multistep Mouse Model for Mammary Carcinogenesis

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Cancers arise through alterations in two general classes of genes, tumor suppressor genes and proto-oncogenes. Because cancers do not appear to develop from the derangement of a single gene, but instead require a series of changes in genes from both of these two groups, cancer development is referred to as "multistep tumorigenesis." For a few systems, such as colon cancer, a reasonable consensus exists as to which mutations occur and in what sequence (Fearon and Vogelstein 1990). Presently, many laboratories are attempting to understand which genetic changes are responsible for breast cancer, one of the most common forms of human cancers.

Mutations of tumor suppressor genes occur frequently in human breast cancer. For example, the retinoblastoma gene (*Rb*) undergoes somatic mutation during the development of some human breast cancers (Weinberg 1992). *p53*, the gene most commonly mutated in all human cancers, is mutated or deleted in at least 22% of sporadic breast tumors (Greenblatt et al. 1994). In addition, some patients with Li-Fraumeni syndrome carry germ-line mutations of *p53* which predispose them to a variety of neoplasms, one of the most common of which is breast cancer (Malkin et al. 1990; Srivastava et al. 1990). When inherited in mutant form, *BRCA1*, a putative tumor suppressor gene on chromosome 17q, renders an individual susceptible to breast cancer. *BRCA1* mutations may be present in as many as 45% of families with a high incidence of early-onset breast cancer and in 80% of families with an increased incidence of early-onset breast and ovarian cancers (Futreal et al. 1994; Miki et al. 1994). Finally, *BRCA2*, recently mapped to human chromosome 13q, may be another tumor suppressor gene responsible for many of the families with early-onset breast cancer that do not show mutation of *BRCA1* (Wooster et al. 1994).

Two other genes when mutated confer an inherited risk for human breast cancer. First, rare mutant alleles of the androgen receptor are associated with male breast cancer (Wooster et al. 1992; Lobaccaro et al. 1993). Second, patients heterozygous for the gene mutated in ataxia telangiectasia are at an increased risk for breast cancer (Swift et al. 1991).

At least three proto-oncogenes have been implicated in human breast carcinogenesis. These include the genes c-*myc*, *neu*, and *PRAD-1/cyclin D1*, each of which has been found amplified and overexpressed in many human mammary tumors (Machotka et al. 1989; Lammie and Peters 1991). Genomic amplification accompanied by overexpression of *neu* in human breast tumors has been correlated with decreased survival (Slamon et al. 1987). The genomic amplicon that includes *PRAD-1/cyclin D1* also includes the linked genes *int-2/Fgf-3* and *hst/Fgf-4*, genes that are involved in mouse mammary tumors (see below), although they are not expressed in human breast tumors (Lammie et al. 1991).

Transgenic mouse models for breast cancer involving c-*myc*, *neu*, *int-2/Fgf-3*, and *PRAD-1/cyclin D1* have been made using the mouse mammary tumor virus (MMTV) or the whey acidic protein promoter/enhancers which direct expression of each transgene to the mammary gland (Cardiff and Muller 1993; Wang et al. 1994). Female transgenic mice from each of these systems develop mammary tumors, although in nearly all cases it is apparent that events in addition to inheritance of the transgene are required to produce a tumor.

In addition to the usefulness of its regulatory signals to produce transgenic models of breast cancer, MMTV itself has proven to be a powerful tool for studying mammary carcinogenesis in the mouse. Infection of mice by MMTV produces mammary adenocarcinomas (Hilgers and Bentvelzen 1979), and the sites of insertion of MMTV proviruses in tumor cell DNA have been studied in an effort to understand the mode of MMTV-induced carcinogenesis. Sequences flanking MMTV integration sites provided probes that were used to isolate neighboring genes and to show that the proviral insertions transcriptionally activate those genes through the enhancer in the MMTV long terminal repeat (LTR) (Nusse and Varmus 1982). The putative proto-oncogenes activated by MMTV proviruses in-

clude two members of the *Wnt* gene family (*Wnt-1* and *Wnt-3*), three members of the fibroblast growth factor (*Fgf*) gene family (*int-2/Fgf-3*, *hst/Fgf-4*, and AIGF/*Fgf-8*), and *int-3*, a mammalian *Notch* gene.

Wnt-1 TRANSGENIC MODEL

The first MMTV integration site identified was *Wnt-1*, the mammalian homolog of the *Drosophila* segment polarity gene, *wingless* (Nusse and Varmus 1982; van Ooyen and Nusse 1984; Rijsewijk et al. 1987b). *Wnt-1* was the first cloned member of an extremely large gene family, with as many as 14 genes in the mouse, and with homologs in all metazoa tested. *Wnt-1* encodes a cysteine-rich, glycosylated secretory protein (Bradley and Brown 1990; Papkoff and Schryver 1990), which is normally expressed during mouse embryonic development and in the round spermatid of the adult testis (Shackleford and Varmus 1987; Wilkinson et al. 1987). Expression of *Wnt-1* is essential for the formation of the brain, since mice deficient in *Wnt-1* lack portions of the midbrain and cerebellum (McMahon and Bradley 1990; Thomas and Capecchi 1990; Thomas et al. 1991). Although *Wnt-1* is not expressed in the normal mammary gland, an exogenous *Wnt-1* gene can transform certain mammary epithelial cell lines in vitro (Brown et al. 1986; Rijsewijk et al. 1987a). Although two *Wnt* genes have been found as MMTV integration sites in mouse mammary tumors, *Wnt* genes have not been implicated thus far in human breast cancer.

To demonstrate that MMTV integrations into the *Wnt-1* locus were instrumental in the development of mammary tumors, transgenic mice were generated in which the transgene mimics an MMTV-activated allele, with the MMTV LTR in the opposite transcriptional orientation to the genomic *Wnt-1* sequence (Tsukamoto et al. 1988). The transgene is expressed principally in the mammary and salivary glands, and all of the mammary glands of both male and female *Wnt-1* transgenic mice (*Wnt-1 TG*) are hyperplastic. Mammary adenocarcinoma eventually develops in all female animals, although usually in only one of the hyperplastic mammary glands. 50% of virgin *Wnt-1 TG* females develop tumors by about 6 months of age (Fig. 1A), whereas only 15% of *Wnt-1 TG* males develop mammary tumors by 1 year of age (Fig. 1B). Metastases occur rarely to the lungs and lymph nodes.

The stochastic nature of tumor development suggests that *Wnt-1* expression is necessary but not sufficient for mammary tumor formation. Additional genetic or epigenetic events probably need to accompany *Wnt-1* expression to form mammary tumors in these transgenic animals. The rather slow kinetics of tumor development have allowed experiments to be designed to ask if this process can be accelerated (Table 1). These have included (1) crossing the *Wnt-1 TG* mice to other genetically altered mice to enhance susceptibility to tumor formation (Kwan et al. 1992; L.A. Donehower et al., in prep.) and (2) infecting the *Wnt-1 TG* mice with MMTV and analyzing new MMTV proviruses to identify genes that might cooperate with *Wnt-1* (Shackleford et al. 1993; C.A. MacArthur et al., in prep.). Such experiments have led to insights into the nature of the proteins that can cooperate with *Wnt-1* in tumor formation.

SYNERGY BETWEEN *Wnt-1* AND FIBROBLAST GROWTH FACTORS

Both genetic crosses between transgenic animals and MMTV infection of *Wnt-1 TG* animals have suggested that members of the *Fgf* gene family cooperate with *Wnt-1* during tumorigenesis. A genetic cross between *Wnt-1 TG* and *int-2/Fgf-3* transgenic animals was inspired by the observation that some MMTV-induced tumors from nontransgenic animals show transcriptional activation of both *Wnt-1* and *int-2/Fgf-3* (Peters et al. 1986). Although *Wnt-1 TG* females readily develop tumors (as described above), mammary tumors occur infrequently in *int-2/Fgf-3* transgenic females and not at all in males (Muller et al. 1990). Females transgenic for both *Wnt-1* and *int-2/Fgf-3* develop tumors faster than animals bearing either transgene alone (Fig. 1A) (Kwan et al. 1992). A more dramatic phenotype is seen in males, with tumors appearing much earlier and in many more animals than in those carrying only the *Wnt-1* transgene (Fig. 1B) (Kwan et al. 1992). The fact that tumors appear earlier in bitransgenic animals than in either parental line suggests that the two genes, *Wnt-1* and *int-2/Fgf-3*, both of which encode secretory proteins, cooperate to form mammary tumors.

This observation was strengthened by experiments in which *Wnt-1 TG* animals were infected with MMTV (Shackleford et al. 1993). MMTV accelerated the kinetics of tumor formation (Figs. 1A,B), and provirus tagging allowed the identification of cooperating genes. Approximately half of the mammary tumors from MMTV-infected *Wnt-1 TG* female mice showed insertional activation of *int-2/Fgf-3*, *hst/Fgf-4*, or both, as well as insertions into AIGF/*Fgf-8* (Shackleford et al. 1993; C.A. MacArthur et al., in prep.), further confirming a strong cooperative interaction between Wnt-1 and members of the Fgf family during mammary carcinogenesis in this model.

EFFECT OF *p53* DEFICIENCY ON *Wnt-1* TUMORIGENESIS

The importance of *p53* as a tumor suppressor gene has been reinforced by genetically engineered mice containing germ-line null alleles of *p53* (Donehower et al. 1992; Jacks et al. 1994; Purdie et al. 1994). Approximately half of the mice that are heterozygous for a null *p53* allele develop sarcomas and lymphomas by 18 months of age, whereas 100% of mice nullizygous for *p53* develop tumors by 10 months of age, principally T-cell lymphomas or, less frequently, sarcomas (Harvey et al. 1993; Jacks et al. 1994). Despite evidence for the

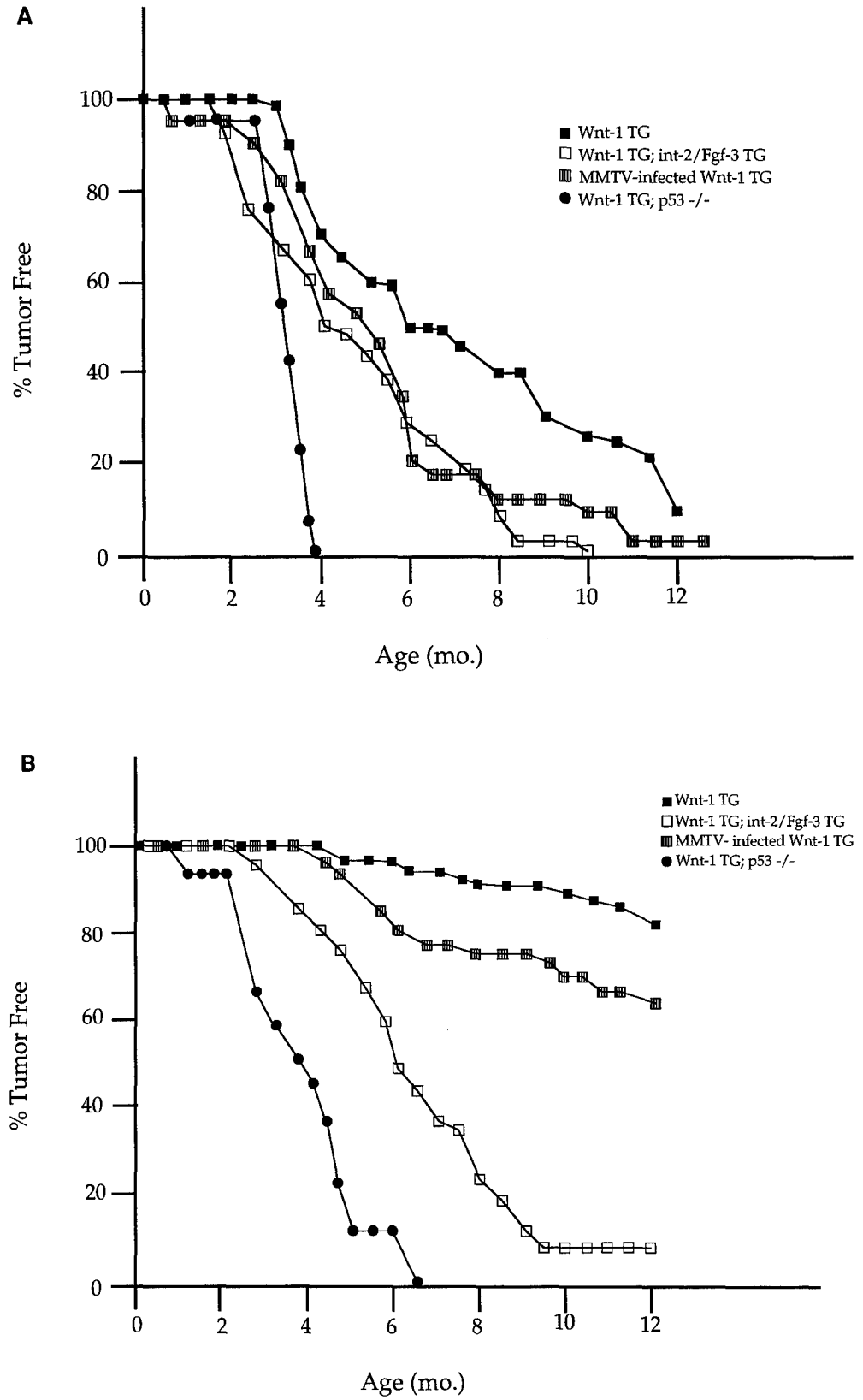

Figure 1. Compiled tumor incidence curves in *Wnt-1 TG* mice from four experiments. The percentage of animals that remained free of mammary tumors is plotted as a function of age. (*A*) *Wnt-1 TG* females; (*B*) *Wnt-1 TG* males. The references for each curve are as indicated: *Wnt-1 TG* mice (Tsukamoto et al. 1988); *Wnt-1 TG*; *int-2/Fgf-3 TG* (Kwan et al. 1992); MMTV-infected *Wnt-1 TG* (Shackleford et al. 1993); *Wnt-1 TG*; p53 − / − (L. Donehower et al., in prep).

Table 1. Experimental Approaches to Find Genes That Cooperate with a Transgene in Tumorigenesis

Experimental approaches	Experiments involving *Wnt-1 TG* mice
1. Cross to other transgenic and gene-targeted mouse lines to look for a change in the kinetics of tumorigenesis	1. Cross with *int-2/FGF3 TG* mice and *p53*-deficient mice; acceleration of tumorigenesis seen in progeny from each cross (cf. Fig. 1)
2. Retroviral infection to search for additional insertionally activated genes	2. MMTV infection accelerates tumorigenesis; proviral insertion mutations of *int-2/Fgf-3, hst/Fgf-4,* AIGF/*Fgf-8*
3. Cross to other mouse strains to look in tumors of F_1 animals for LOH throughout the mouse genome (Dietrich et al. 1992)	3. Cross with *M. castaneus*; results pending on tumors from F_1 transgenics
4. Use PCR-based methods to clone sequences related to a gene family of interest that might be implicated in mammary tumorigenesis	4. Identification of *Sky,* a transmembrane protein-tyrosine kinase, expressed in C57MG cells and mammary tumors
5. Differential display or subtractive hybridization to seek genes differentially expressed during stages of mammary tumorigenesis	5. Identification of gene for demilune cell-specific protein, a putative secretory protein, expressed in mammary gland hyperplasia

On the left, we present the general approaches that can be taken to identify genes which cooperate with a given transgene. On the right, we present the application of these approaches that we have used in the study of the *Wnt-1* transgenic mice.

involvement of *p53* in human breast cancer, these mice rarely develop mammary tumors.

To seek an effect of *p53* loss on mammary tumorigenesis, we crossed *p53*-deficient mice to the *Wnt-1 TG* mice, which are predisposed to breast cancer (L.A. Donehower et al., in prep.). The F_1 and F_2 offspring from these crosses fell into twelve categories, depending on sex, *p53* genotype, and the presence or absence of the *Wnt-1* transgene. Mammary tumors appeared in all of the female mice with the *Wnt-1* transgene before 1 year of age, regardless of *p53* status (Table 2). *Wnt-1 TG p53* + / + and *p53* + / − females developed mammary tumors at similar rates, with an average age of 22.5 weeks and 23 weeks of age, respectively (Table 2, column 2). In contrast, the average age of onset of mammary carcinomas in *Wnt-1 TG* females nullizygous for *p53* was 11.5 weeks of age, and all of these mice formed tumors by 15 weeks (Fig. 1A and Table 2, columns 2 and 3).

Male *Wnt-1 TG* mice wild-type or heterozygous for *p53* showed modest and not significantly different rates of mammary tumor development by 1 year of age (32% and 43%, respectively). In contrast, mammary tumors appeared in *Wnt-1 TG p53* − / − males at a rate almost as rapid as that observed in females of the same genotype (Fig. 1B). Tumors appeared in all of the *Wnt-1 TG p53* − / − males by 6 months of age. Of these, 75% were mammary tumors and 25% were lymphomas and sarcomas similar to those found in the *p53* − / − mice without the *Wnt-1* transgene. (Animals with non-mammary tumors are not included in Fig. 1B.)

Male and female mice without the *Wnt-1* transgene, but either heterozygous or homozygous for a *p53* null allele, developed a variety of non-mammary tumors with incidences comparable to those described previously (Donehower et al. 1992). Mice without the *Wnt-1* transgene and with two wild-type *p53* alleles did not form any tumors during the monitoring period.

LOSS OF HETEROZYGOSITY IN *Wnt-1 TG p53* + / − MAMMARY TUMORS

DNA isolated from tumors arising in *Wnt-1 TG* mice heterozygous for a null *p53* allele was examined for retention or loss of the remaining wild-type allele. Approximately half of *Wnt-1 TG p53* + / − tumors showed loss of the wild-type *p53* allele (data not shown), implying selection for the growth of cells lacking *p53* during tumor development. However, tumors which displayed loss of heterozygosity (LOH) of the

Table 2. Incidence, Histopathology, and Chromosomal Instability of Tumors from Female *Wnt-1* Transgenic Mice in the Presence or Absence of *p53*

p53 Genotype	50% Tumor incidence[a] (weeks)	100% Tumor incidence[b] (weeks)	Tumors with fibrosis[c]	Aneuploid tumors[d]	Tumors with chromosome abnormalities[e]	Average number of chromosome abnormalities per tumor[f]
p53 +/+	22.5	40	8/11	0/5	2/6	0.3
p53 +/− (no LOH)	23.0[g]	41[g]	6/8	0/1	2/4	1.0
p53 +/− (LOH)			1/7	2/3	8/8	4.2
p53 −/−	11.5	15	0/6	3/6	7/7	1.7

[a] Age by which 50% of all mice of a given *p53* genotype had developed mammary tumors.

[b] Age by which 100% of all mice of a given *p53* genotype had developed mammary tumors.

[c] Number of tumors with extensive regions of fibrosis following histopathology/number of tumors of that *p53* genotype analyzed.

[d] Number of tumors with >40% of cells displaying aneuploid karyotype by standard cytogenetics/number of tumors of that *p53* genotype analyzed.

[e] Number of tumors of a group with abnormal CGH profiles/number of tumors of that *p53* genotype analyzed.

[f] Average number of CGH copy number increases and decreases per tumor of a given *p53* genotype.

[g] Not all *Wnt-1 TG/p53* +/− tumors were assayed for *p53* LOH; 23 and 41 weeks are the ages at which 50% and 100% of all *p53* +/− mice developed mammary tumors, respectively.

wild-type *p53* allele did not appear earlier on average than *Wnt-1 TG p53* + / − mammary tumors that retained the wild-type *p53* allele. *p53* cDNAs from four of the *Wnt-1 TG p53* + / − mammary tumors without *p53* LOH were sequenced and found to have the normal nucleotide sequence.

HISTOPATHOLOGY OF MAMMARY TUMORS DEPENDS ON *p53* GENOTYPE

Dramatic histopathological differences were noted between those tumors that contained one or two wild-type *p53* genes and those without a wild-type *p53* gene. Tumors that had at least one wild-type *p53* allele tended to be more organized, fibrotic, and more differentiated, whereas tumors that lacked wild-type *p53* (*p53* − / −, and *p53* + / − with *p53* LOH) were significantly more anaplastic, less differentiated, and less fibrotic (Table 2, column 4).

MEASURING GENOMIC INSTABILITY IN *Wnt-1 TG* MAMMARY TUMORS

We were interested in understanding some of the mechanisms by which the lack of *p53* affected mammary tumorigenesis in the *Wnt-1 TG* mice. Previous studies have indicated that wild-type p53 may operate as a cell cycle checkpoint protein that mediates G_1 arrest or apoptosis following DNA damage or abnormal proliferation (Kastan et al. 1991, 1992; Kuerbitz et al. 1992; Clarke et al. 1993; Lowe et al. 1993, 1994). Models have been proposed in which loss of wild-type *p53* leads to a loss of cell cycle control and consequently, genomic instability (Lane 1992). Increased rates of DNA rearrangements or other mutations could contribute to oncogenesis. Support for such a model has been provided by Yin et al. (1992) and Livingstone et al. (1992), who showed that cells in culture that lose wild-type *p53* have higher levels of genomic instability as measured by more abnormal chromosome numbers and increased susceptibility to gene amplification under selective conditions. To test whether *p53* deficiency predisposes cells to increased genomic instability in the context of an animal tumor model, we examined the mammary tumors from the study described above by three methods: standard karyotype analysis, comparative genomic hybridization, and Southern blotting.

KARYOTYPES OF CULTURED CELLS FROM *Wnt-1 TG* MAMMARY TUMORS

In the karyotyping analyses, we examined chromosome numbers from cultured cells derived from fifteen *Wnt-1 TG* mammary tumors of different *p53* genotypes. Of five *p53* + / + tumors analyzed, all contained tumor cells that displayed predominantly diploid karyotypes (Table 2, column 5). This was also true of one *p53* + / − tumor which retained its wild-type *p53* allele and of one *p53* + / − tumor which displayed *p53* LOH. In contrast, two of three *p53* + / − tumors with *p53* LOH and three of six *p53* − / − tumors had a high percentage of cells with aneuploid karyotypes (Table 2, column 5). The remaining three *p53* − / − tumors had karyotypes that resembled those from *p53* + / + tumors. Thus, lack of *p53* was necessary for a non-diploid karyotype, but was not sufficient for it. This confirms that *p53* deficiency predisposes cells in this tumor model to increased genomic instability at the chromosomal level.

COMPARATIVE GENOMIC HYBRIDIZATION

Comparative genomic hybridization (CGH) detects regions of increased and decreased DNA copy number throughout the entire genome (Kallioniemi et al. 1992). Application of this technique to the *Wnt-1 TG* mammary tumors showed that loss of *p53* produced increased genomic instability at the chromosomal and subchromosomal level. Four of six *p53* + / + and two of four *p53* + / − mammary tumors with retention of wild-type *p53* displayed normal CGH profiles (Table 2, column 6). Each of the two *p53* + / + tumors with abnormal CGH profiles exhibited a DNA copy number change in only a single chromosomal region (Table 2, column 6). Two of four *p53* + / − tumors that retained wild-type *p53* had CGH abnormalities of one and three chromosomal regions each. In contrast, the *p53* + / − tumors with *p53* LOH showed the highest levels of chromosomal abnormalities. All eight of these tumors displayed abnormal CGH profiles, with an average of 4.2 abnormalities (mostly DNA copy number decreases) per tumor (Table 2, columns 6 and 7). Finally, all seven *p53* − / − tumors displayed CGH abnormalities, with a mean number of abnormalities per tumor of 1.7 (Table 2, columns 6 and 7).

For the 21 tumors analyzed by CGH, the frequency with which each chromosome contained a region of DNA gain or loss is displayed graphically in Figure 2. Note that chromosomes 8, 9, 11, 13, 14, and X showed frequent loss of DNA sequences. The losses on chromosome 11 were mostly observed in the *p53* + / − tumors that lost wild-type *p53*. This result is consistent with the location of the *p53* gene on mouse chromosome 11. Interestingly, a number of the other implicated regions contain genes that have been associated with mammary cancer in humans or in other mouse models (Fig. 2, right-hand panel).

Southern blotting of normal and mammary tumor DNA from *Wnt-1 TG/p53* − / − animals was used to test for DNA copy increases of specific loci. The loci that were chosen were three that are amplified in some human breast cancers: c-*myc*, *neu*, and *int-2/Fgf-3* (Machotka et al. 1989; Lammie and Peters 1991). Such analysis revealed one tumor (W177B) with an increased DNA copy number at the *int-2/Fgf-3* locus. This result was consistent with the CGH profile for that tumor, which showed a DNA increase in the telomeric region of mouse chromosome 7, the chromosomal localization of *int-2/Fgf-3*. Quantitation of the South-

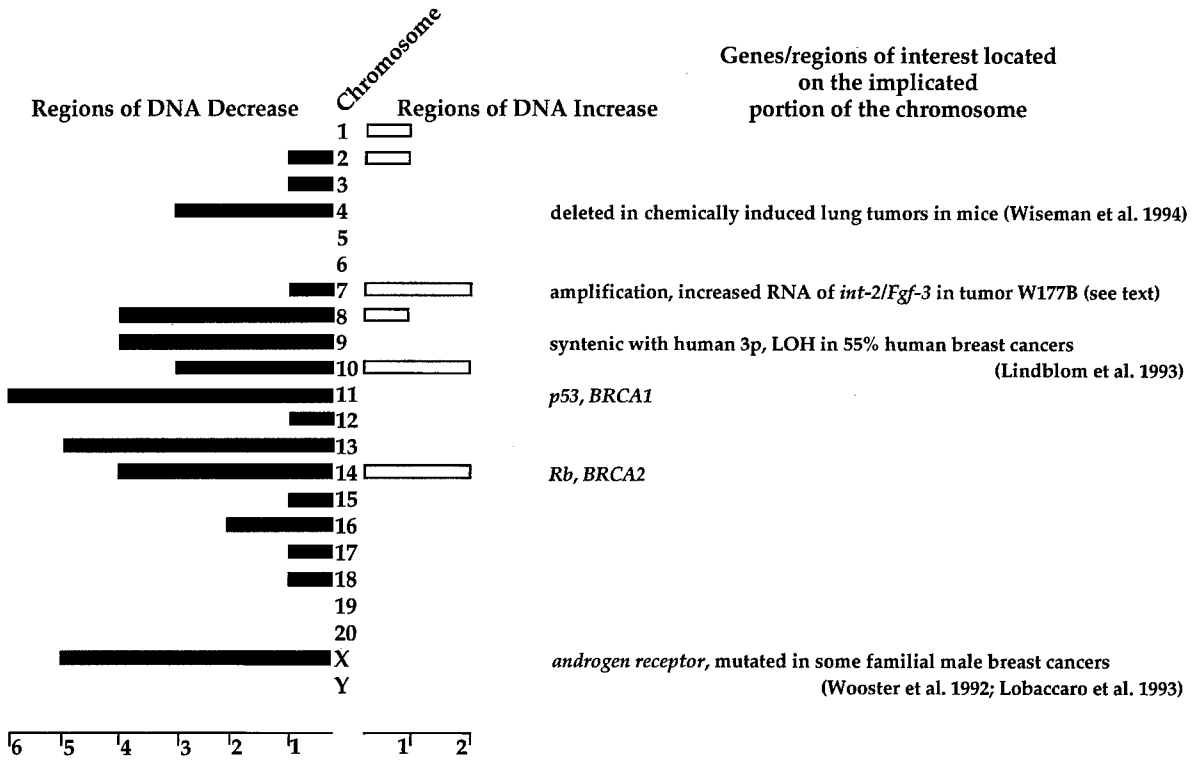

Figure 2. Summary of the chromosomal changes seen by CGH in *Wnt-1*-induced mammary tumors. The length of each solid bar indicates the number of tumors for which a decrease in DNA copy number was found on that chromosome. The length of each outlined bar indicates the number of tumors for which an increase in DNA copy number was found on that chromosome. Genes located in the implicated region which might play a role in tumor formation are listed on the right of the figure.

ern blot revealed that this locus was present in 6–8 copies, and Northern blot analysis showed abundant *int-2/Fgf-3* RNA as well. However, in human breast tumors with DNA increases at this locus, *int-2/Fgf-3* RNA is not detectable, and the DNA amplification appears to contribute to tumorigenesis by expression of the linked *PRAD-1/cyclin D1* locus (Lammie et al. 1991). *PRAD-1/cyclin D1* was not expressed at augmented levels in tumor W177B, suggesting that selective pressures may favor expression of different genes, such as *int-2/Fgf-3*, in the *Wnt-1* transgenic context.

Taken together, the CGH results confirm the increased genomic instability in tumors lacking *p53* at the chromosomal and subchromosomal level. In addition, recurring regions of chromosomal loss and gain suggest that the observed genomic instability may not be random but may reflect selection for genetic changes that promote tumor progression. The increased levels of *int-2/Fgf-3* RNA in one *Wnt-1 TG p53* − / − tumor, for example, are consistent with the known synergy between Wnt-1 and fibroblast growth factors in mammary carcinogenesis.

OTHER APPROACHES TO FINDING *Wnt-1* COOPERATING GENES

Since loss of heterozygosity of tumor suppressor genes is a frequent event during tumorigenesis, interspecies crosses can be used to search the entire genome for loci which have undergone LOH (Dietrich et al. 1992; Copeland et al. 1993). In such an interspecies cross, the F_1 animals have completely heterozygous genomes. By examining the heterozygosity profile in F_1-derived mammary tumors as compared to other somatic tissues, the entire genome can be scanned for regions that have undergone LOH. Such an analysis is currently under way using mammary tumors from F_1 animals resulting from a cross between *Wnt-1 TG* and *Mus castaneus* mice in collaboration with Karen Hong and Eric Lander at the Massachusetts Institute of Technology.

The stochastic nature of tumor development in the *Wnt-1 TG* mice suggests that other genetic or epigenetic events must accompany *Wnt-1* overexpression to lead to tumor formation. The ability to separate tumor development into stages of hyperplasia, tumor, and metastasis should allow the identification of genes whose expression is necessary for tumor progression. One approach to finding genes that might be functionally relevant in the development of a particular stage is to identify genes that are differentially expressed among these different stages. Two genes have been identified which show differential expression within the *Wnt-1* transgenic system. They are *Sky*, expressed in mammary tumors, but not in hyperplastic glands, and the gene for demilune cell-specific protein, expressed in hyperplastic glands, but not in mammary tumors.

EXPRESSION OF *SKY* IN MAMMARY TUMORS

As part of our efforts to identify the still unknown Wnt-1 receptor, we used a polymerase chain reaction (PCR)-based strategy to isolate receptor tyrosine kinases in the mouse mammary epithelial cell line C57MG. We reasoned that the Wnt-1 receptor may be a tyrosine kinase, since these types of signaling molecules are key players in the control of cell growth, differentiation, and division. C57MG cells are presumed to express the Wnt-1 receptor, since they undergo morphological transformation, divide past confluence, and form foci in the presence of Wnt-1 (Brown et al. 1986).

Our screen identified five cDNA clones encoding receptor tyrosine kinases for which the ligand is known (fibroblast growth factor receptor, platelet-derived growth factor receptor, epithelial growth factor, insulin receptor, insulin-like growth factor receptor), two putative receptor tyrosine kinases for which the ligand remains to be identified (the products of *ryk* and the mouse *klg* homolog), and a novel tyrosine kinase. We isolated cDNAs encoding both the murine and human homologs of this kinase, the sequences of which were subsequently published under the names *Sky* (Ohashi et al. 1994) and *Rse* (Mark et al. 1994).

Our analysis (I.C.A. Taylor et al., in prep.) has revealed that mouse *Sky* RNA levels are abundant in mammary tumors derived from transgenic mice that express *Wnt-1*, *int-2/Fgf-3*, or both oncogenes in their mammary glands. However, little or no expression of *Sky* is detected in mammary glands from virgin animals or in preneoplastic mammary glands from *Wnt-1 TG* mice. Moreover, the human homolog of *Sky* is expressed at elevated levels when normal human mammary epithelial cells are rendered tumorigenic by the introduction of two viral oncogenes, v-*Ha-ras* and SV40 large T antigen. Transient transfection of a *Sky* expression vector into the quail fibrosarcoma cell line QT6 demonstrated that Sky is an active tyrosine kinase that is glycosylated and forms dimers, characteristics that are hallmarks of the receptor class of tyrosine kinases. The introduction of *Sky* into NIH-3T3 and Rat B1a fibroblasts results in morphological transformation, growth in soft agar, and tumor formation when injected into nude mice, suggesting that overexpression of *Sky* may be sufficient to promote tumorigenesis. Although it remains to be determined whether Sky is the receptor for Wnt-1, its expression pattern and transforming properties in cell culture raise the intriguing possibility that it is involved in the development and/or progression of mammary tumors.

DIFFERENTIALLY EXPRESSED GENES

Genes that are differentially expressed may be functionally significant for the development of a particular stage of tumor development. One technique designed to identify such genes, named differential display (Liang and Pardee 1992), allows the resolution of reverse transcriptase (RT)-PCR products on a sequencing gel. By comparing the cDNAs generated with RNAs from nontransgenic mammary gland, *Wnt-1 TG* hyperplastic gland, *Wnt-1 TG* mammary tumor, or metastatic foci, candidate cDNAs are found which appear to be differentially expressed. These cDNAs are then used as probes on Northern blots to confirm differential expression.

One such cDNA has been identified and is expressed in *Wnt-1 TG* hyperplastic glands but not in tumors. The sequence of this cDNA matched that of an unpublished cDNA that had been cloned from a mouse salivary gland cDNA library (I. Bekhor et al., in prep.). This cDNA encodes demilune cell-specific protein, a protein with a primary sequence of 170 amino acids, which is highly expressed in the demilune cells of the mouse salivary gland. Although its sequence is not homologous to any protein of known function, the presence of a canonical signal sequence and the absence of a predicted transmembrane domain suggest that it is secreted. The significance of its expression for *Wnt-1*-induced hyperplasia for tumor formation is under investigation.

SUMMARY AND PROSPECTS

The *Wnt-1* transgenic system has provided a rich model for studying mammary tumorigenesis in the mouse. Crossing these animals to other transgenic animals and infecting them with MMTV have forcefully demonstrated the synergy between Wnt-1 and members of the Fgf family. Mammary tumors from *Wnt-1 TG/p53*-deficient mice present novel features: a less fibrotic histology, rapid kinetics of tumor formation, and increased genomic instability. Understanding the molecular basis for such phenotypes may provide insights into the behavior of human tumors, especially those in which *p53* has been affected.

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